Emerging Fluorochrome Technology For Flow and Image Cytometry

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http://home.ncifcrf.gov/ccr/flowcore/ISAC2006/ISAC2006.htm

Polychromatic flow cytometry is now almost routine…

The hardware is available…

…multicolor cytometers like the BD LSR II and FACSAria, Beckman-Coulter FC500, Dako CyAn and Partec CyFlow.

The software is available…

…software packages like DiVa, FlowJo, FCSExpress and DakoCytomation Summit can handle many parameters, software-based compensation, etc.

And the fluorescent reagents are available…

…new probes for flow cytometry, enhanced versions of existing probes (such as the Alexa Fluor dyes)

… and most importantly, many direct conjugates now available with even the more exotic fluorochromes

Polychromatic flow cytometers

- More colors than traditional instruments – from 5 to 20 fluorochromes simultaneously
- BD LSR II, Beckman-Coulter FC500, DakoCytomation CyAn, Partec CyFlow
- More lasers, more detectors
- Not only do more colors, but also probes not possible on other benchtop instruments


BD LSR II

Partec CyFlow


DakoCytomation CyAn

BD Biosciences LSR II

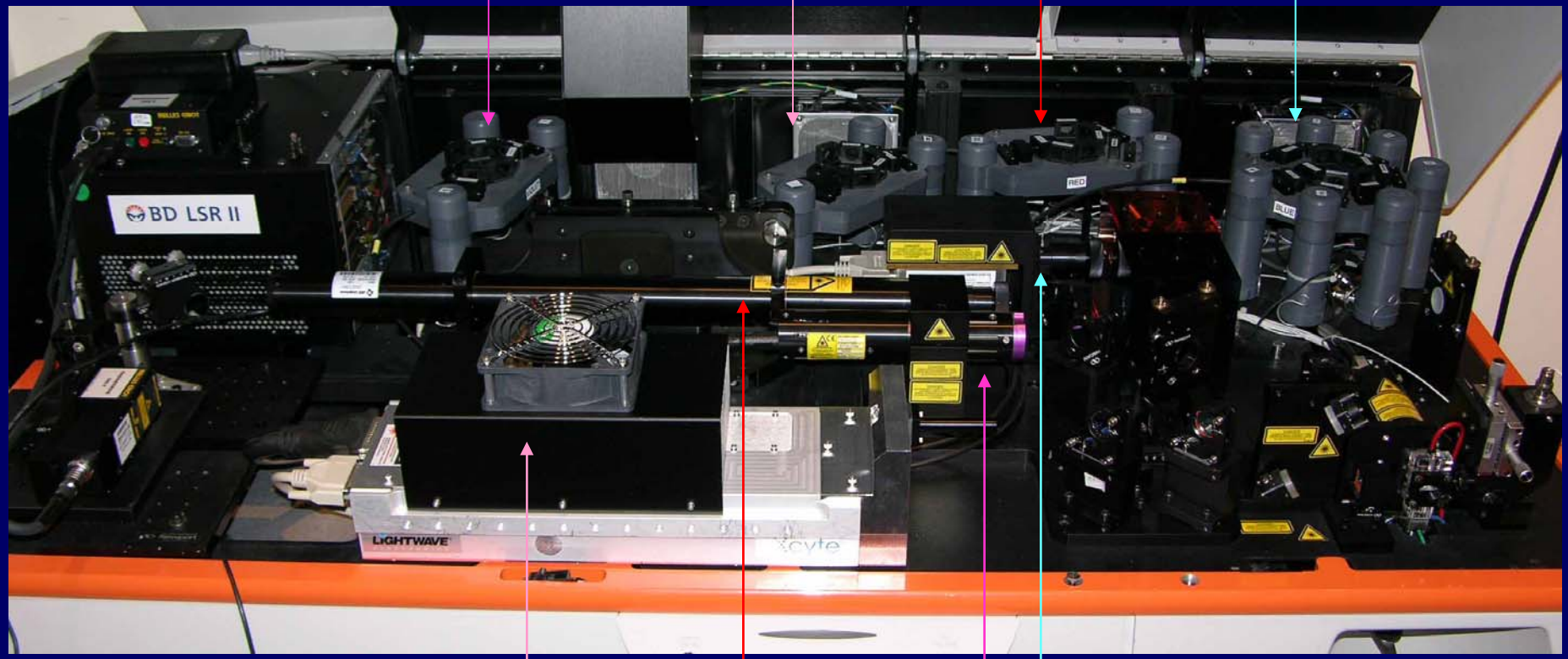
13 detectors

Two detectors off the violet diode

Two detectors off the UV

Three detectors off the red HeNe

Up to 6 detectors off the 488 nm laser



Four lasers

Red HeNe 633 nm

DPSS 488 nm

Nd:YVO_4 UV 355 nm (a small UV laser!)

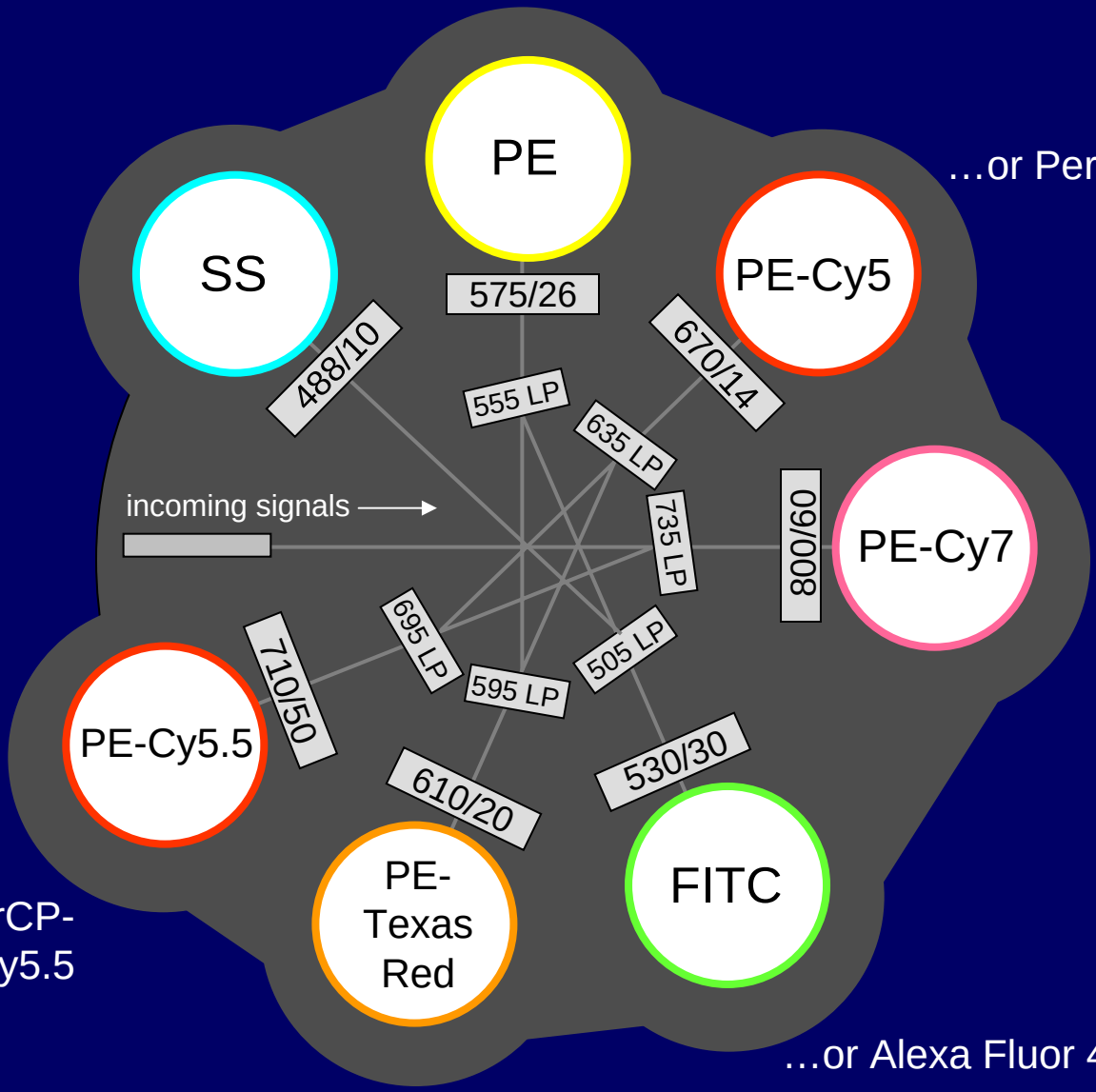
Violet diode 405 nm

“Typical” detector configuration

BD LSR II

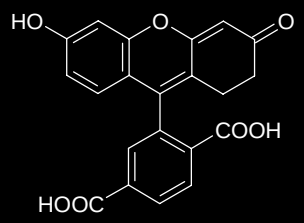
488 nm laser

Fluorescein
Phycoerythrin (PE)
PE-Texas Red
PE-Cy5
PE-Cy5.5
PE-Cy7

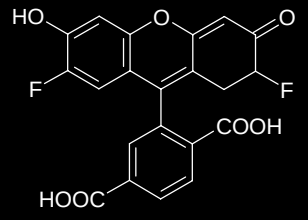


Photostable fluorescein derivatives

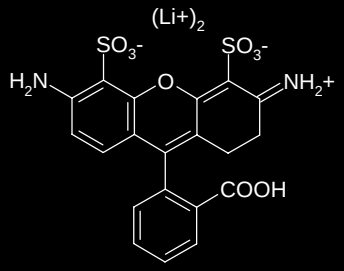
6-carboxyfluorescein

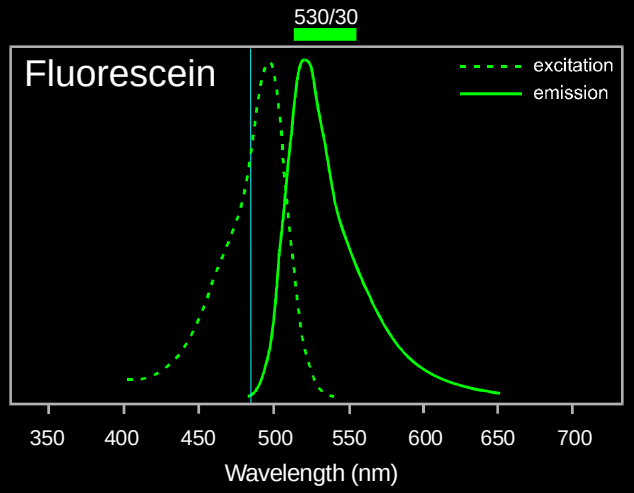


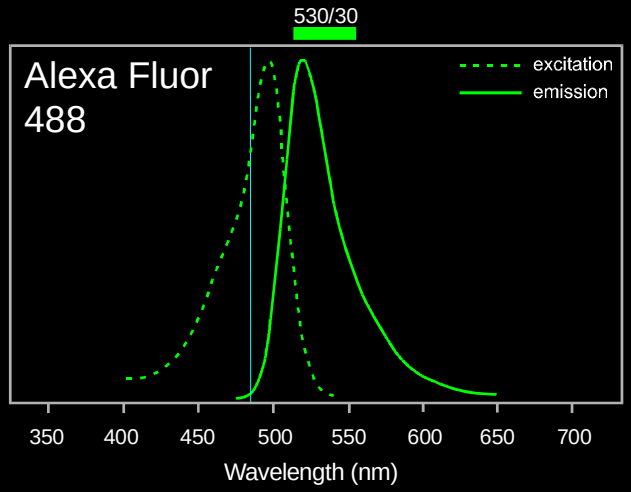
6-carboxy-2',7'-difluoro-fluorescein
(Oregon Green 488™)



Alexa Fluor 488™



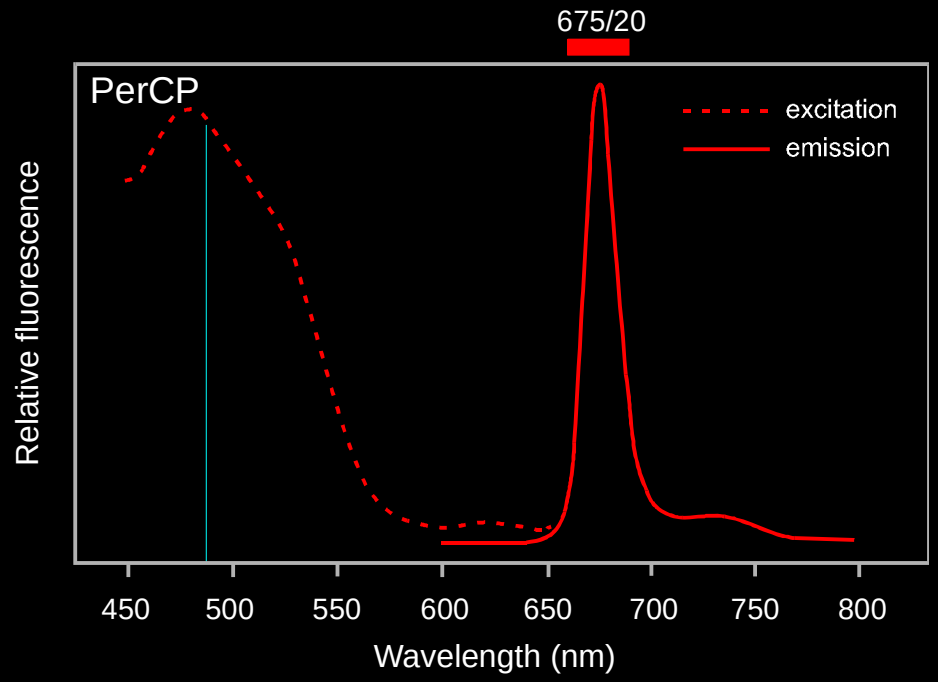




Alexa Fluor 488 direct conjugates are now widely available from several sources.

PerCP (peridinin chlorophyll protein)

- derived from a photosynthetic dinoflagellate protozoan
- much more restrictive emission bandwidth than PE-Cy5 – very little spectral overlap into PE, and no crossbeam compensation issues with APC
- not appreciably excited by red laser light, therefore requiring little crossbeam compensation when used with red-excited fluorochromes
- not particularly bright
- available as a somewhat brighter tandem, PerCP-Cy5.5
- photobleaches and/or converts to a long-lived triplet excitation state upon high-power excitation, limiting its use with higher power water-cooled lasers and cell sorting (and on some newer multicolor analyzers with more powerful lasers)



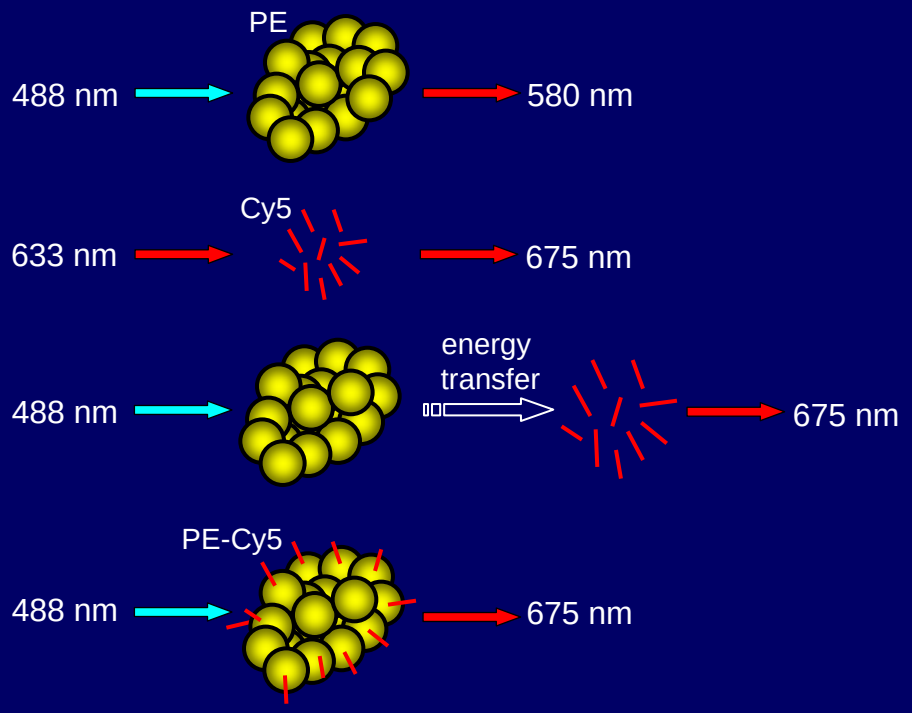
Tandem conjugates

Resonance energy transfer complexes of large protein fluorochromes (such as phycoerythrin or allophycocyanin) and low molecular weight fluorochromes (such as the Cy or Alexa Fluor probes).

- PE-Texas Red
- PE-Cy5
- PE-Cy5.5
- PE-Cy7
- APC-Cy5.5
- APC-Cy7
- PerCP-Cy5.5

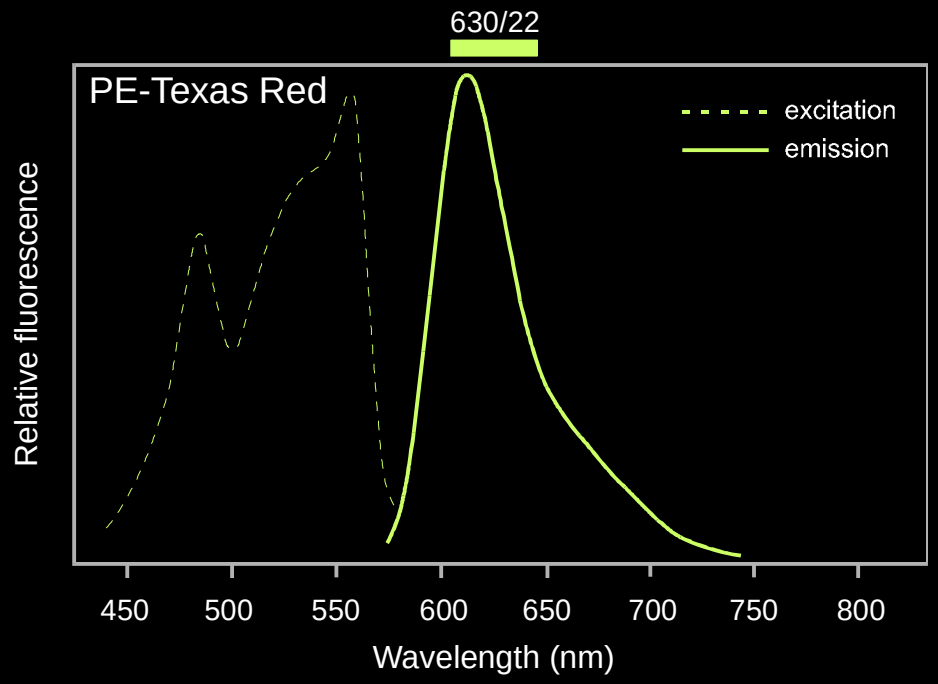
- PE-Alexa Fluor 610
- PE-Alexa Fluor 647
- PE-Alexa Fluor 680

- APC-Alexa Fluor 680
- APC-Alexa Fluor 700
- APC-Alexa Fluor 750



PE-Texas Red (ECD) and PE-Alexa Fluor 610

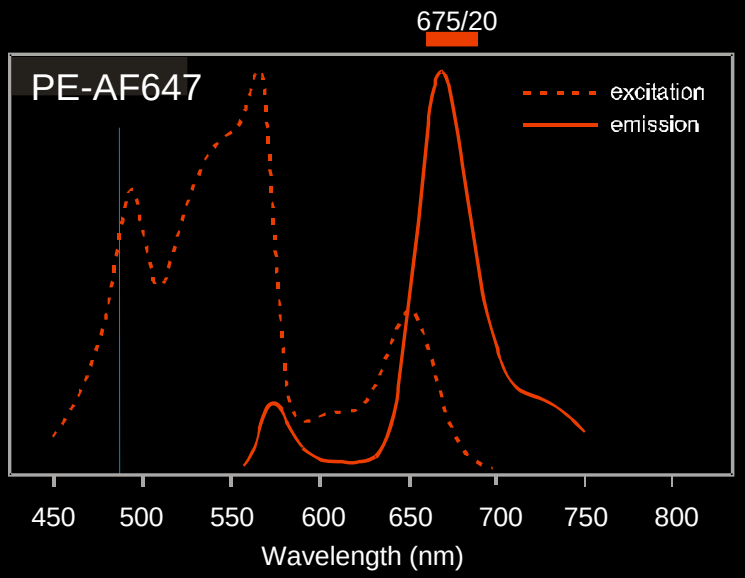
- one of the first commercially available tandem conjugates
- extremely bright – relatively good energy tranfer efficiency
- particularly useful on instruments specially configured for its use, including the XL and the Compucyte LSC
- PE-Alexa Fluor 610 – a PE-Texas Red analog developed by Molecular Probes Invitrogen



PE-Texas Red emission at approximately 610 nm, PE-Alexa Fluor 610 at approximately 625 nm

PE-Cy5 and PE-Alexa Fluor 647

- PE-Cy5 by far the most common tandem conjugate used in flow cytometry
- Sold under far too many trade names (CyChrome, TriColor, Quantum Red, PC5, etc., etc.)
- Current preparations generally show excellent energy tranfer between the PE phycobiliprotein and its Cy5 hapten, usually requiring minimal compensation between PE-Cy5 and PE
- HOWEVER…All lots of PE-Cy5 differ somewhat from one another due to the variability of PE:Cy5 binding ratios and binding sites

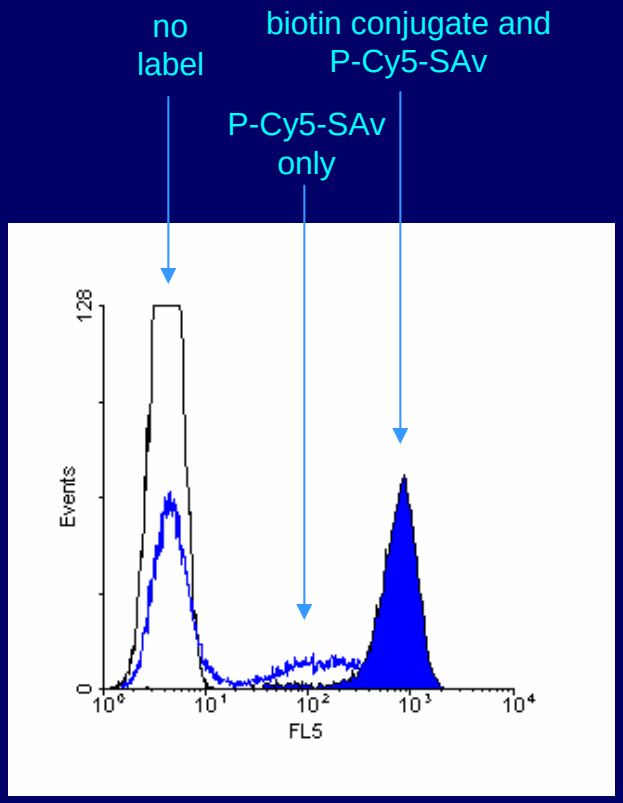


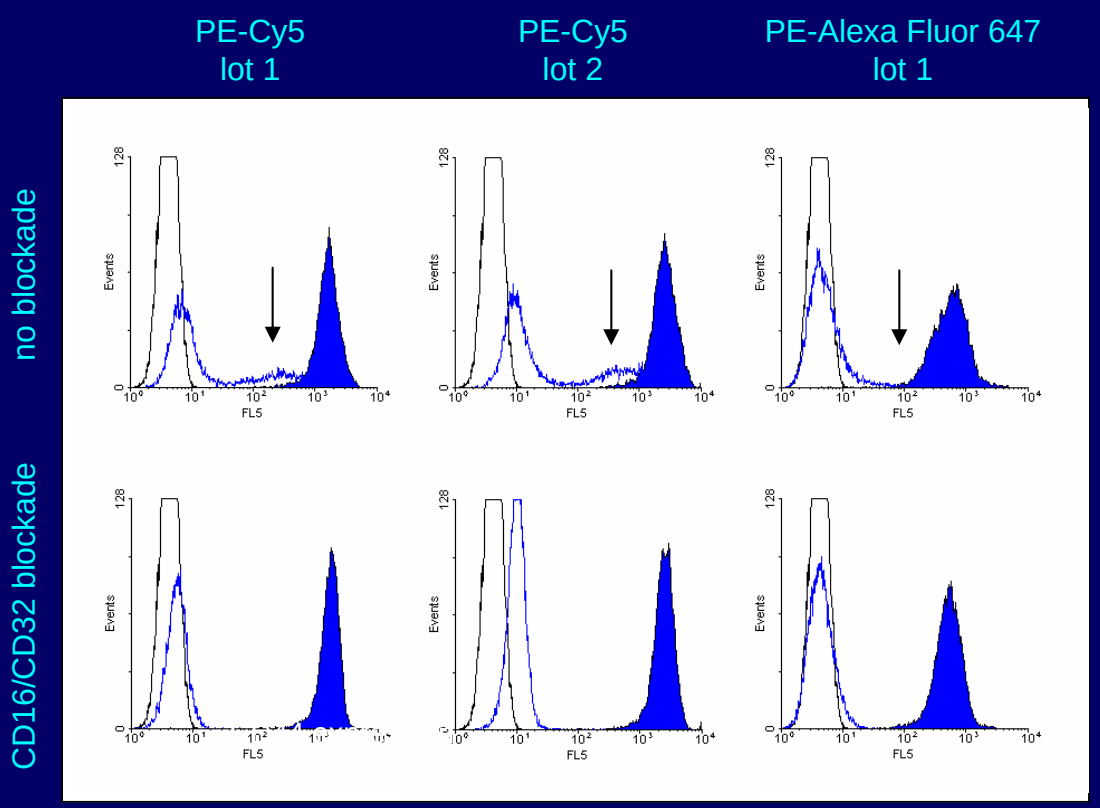
Proper compensation controls using the actual conjugate (not another reagent or bead control are CRITICAL for good fluorescence compensation of PE-Cy5

- Non-specific binding of monocytes/macrophages by Cy fluors
- Not as big a problem as it used to be (due to improved tandems), but still needs to be monitored!

Non-specific Fcγ2/γ3 receptor binding by PE tandem conjugates

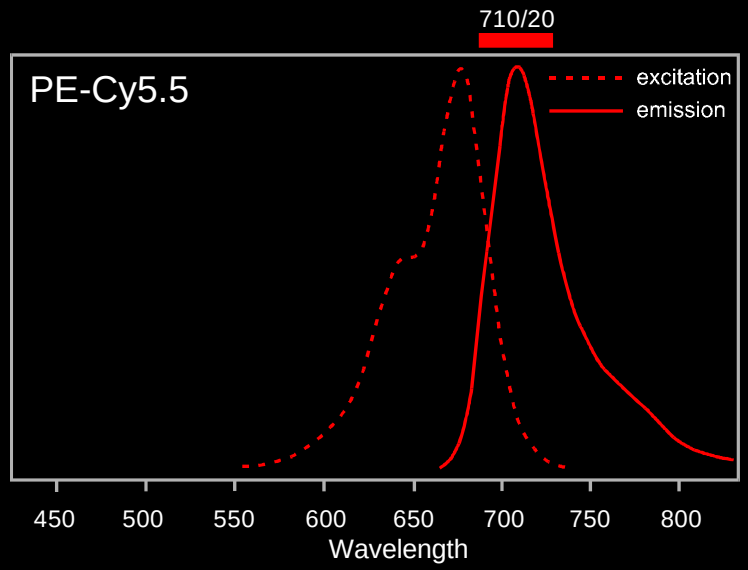
Despite recent improvements, many manufacturers PE-Cy5 conjugates still show high levels of mouse Fcγ2/γ3 binding. Interestingly, PE-Alexa Fluor 647 shows lower levels of this non-specific Interaction than PE-Cy5.

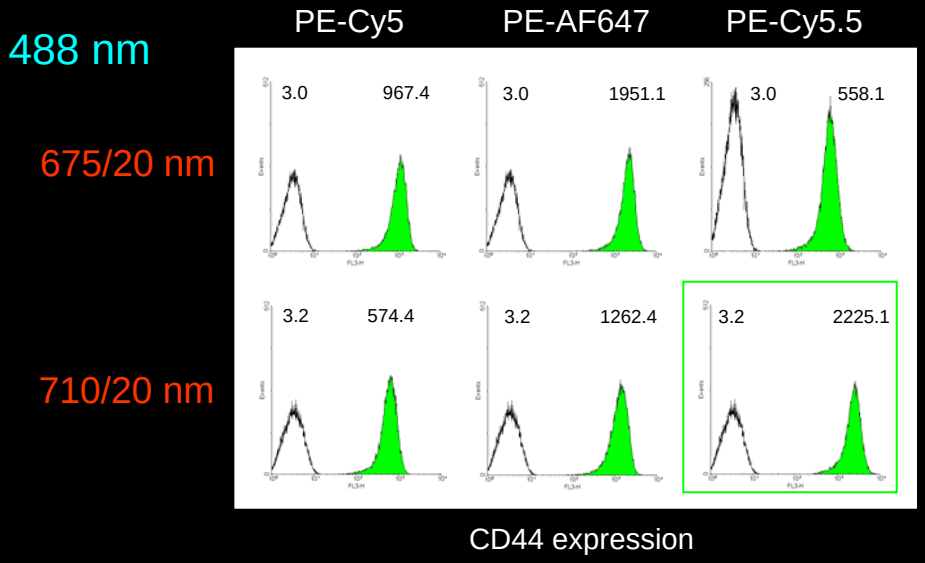




PE-Cy5.5, PE-Alexa Fluor 680 and PE-Alexa Fluor 700

- intermediate emission between PE-Cy5 and PE-Cy7
- PE-Cy5.5 is not particularly new, but now commercially available
- extremely bright – relatively good energy transfer efficiency
- PE-Cy5.5 substitute for PE-Cy5 in applications requiring less intralaser compensation with PE or interlaser compensation with APC
- useful in multicolor labeling with PE and other tandem conjugates
- PE-Alexa Fluor 680 – a PE-Cy5.5 analog developed by Molecular Probes – spectrally interchangeable with PE-Cy5.5
- PE-Alexa Fluor 700 – longer wavelength than PE-Cy5.5 and PE-Alexa Fluor 680



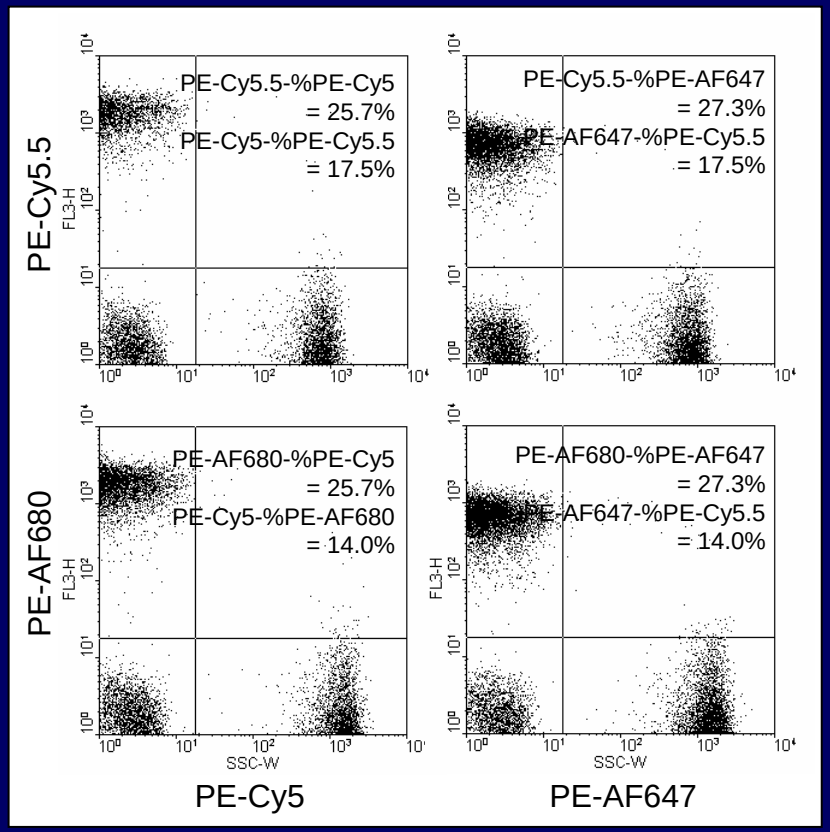


CD44 expression

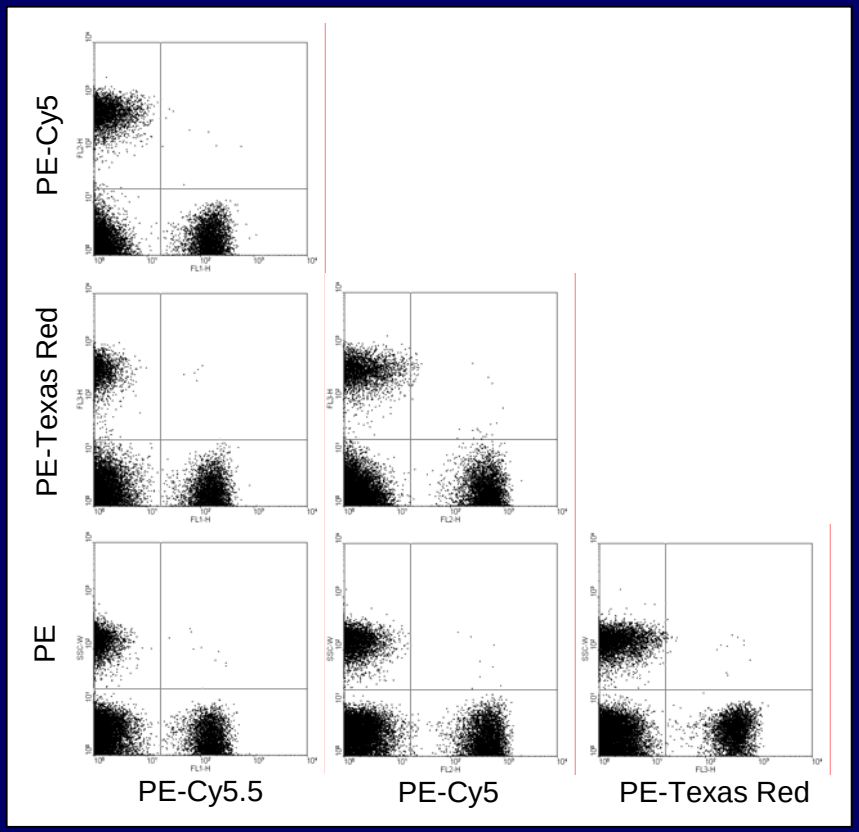
PE-Cy5.5 in multicolor labeling

PE-Cy5.5 and PE-Alexa Fluor 680 combine well with PE and other tandems

PE-Cy5.5 or PE-Alexa Fluor 680
and
PE-Cy5 or PE-Alexa Fluor 647

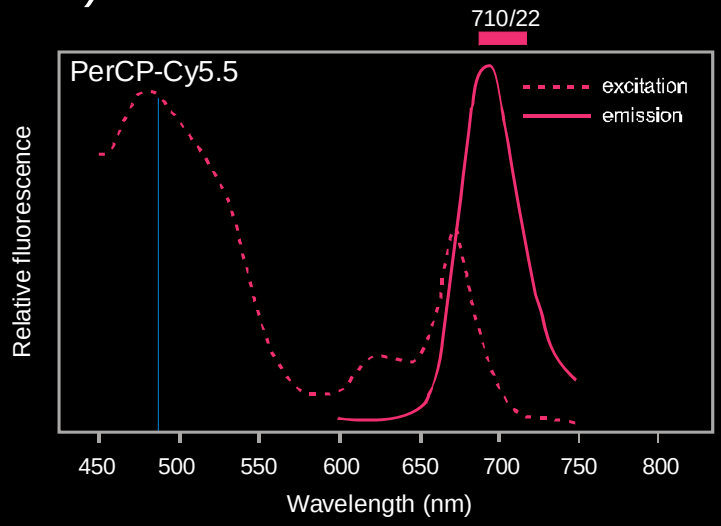


PE
PE-Texas Red
PE-Cy5
PE-Cy5.5



PerCP-Cy5.5 (peridinin chlorophyll protein)

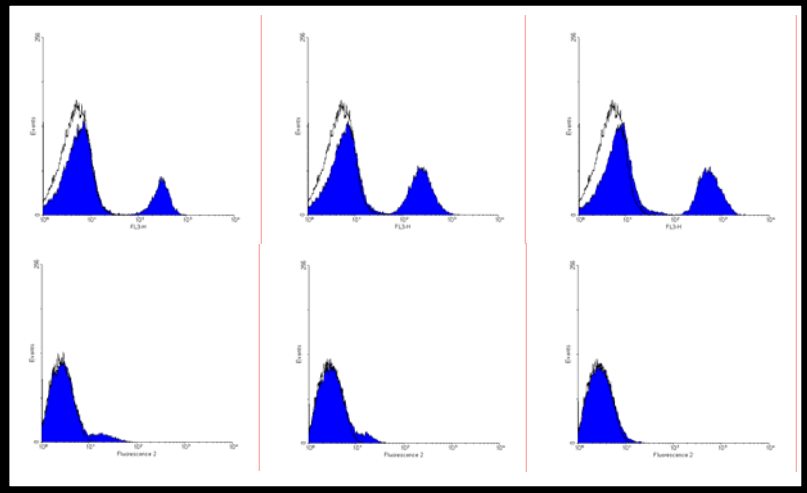
- a PerCP tandem conjugate with Cy5.5
- since it is a tandem conjugate, it does not suffer from the triplet state conversion phenomenon
- However, it also loses fluorescence rapidly with high-power laser excitation, suggesting that PerCP photosensitivity may have multiple mechanisms



PerCP-Cy5.5 emission at approximately 710 nm

FACSCalibur
EX = 488 nm
15 mW
sheath = 5 psi

FACSVantage
EX = 488 nm
50 mW
sheath = 30 psi



mouse EL4 cells

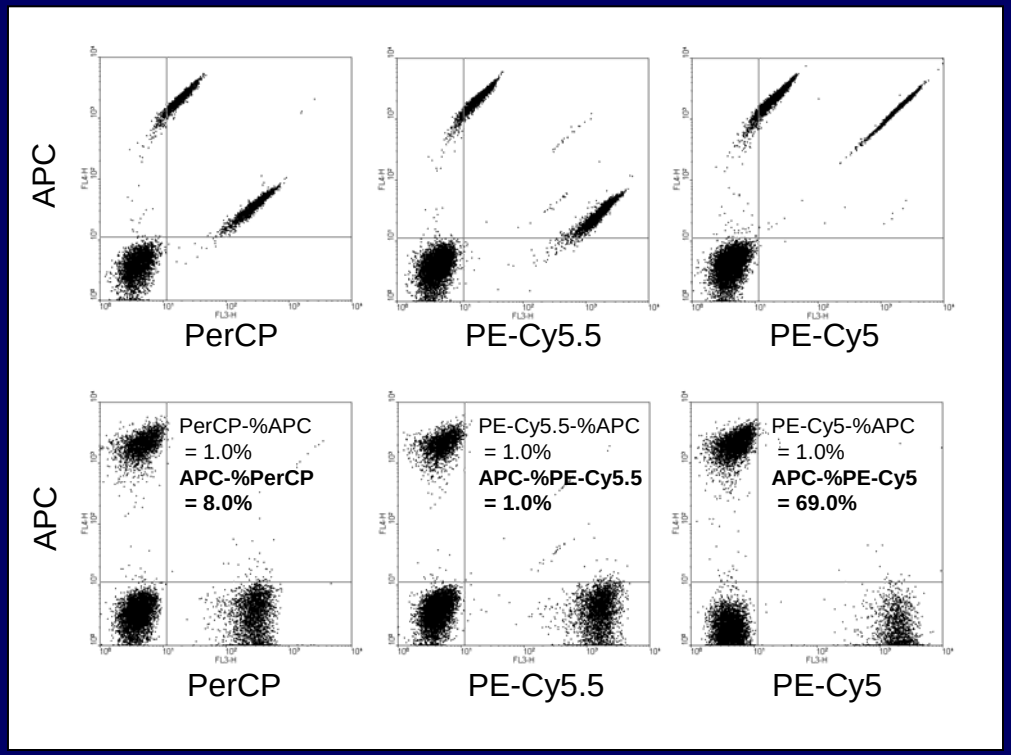
PerCP-CD20 | PerCP-Cy5.5-CD19 | PerCP-Cy5.5-CD20

Crossbeam compensation requirements between APC and PE-Cy5, PE-Cy5.5 and PerCP

A significant problem with simultaneous use of PE-Cy5 and APC is the incidental excitation of PE-Cy5 by the red laser source, requiring a high level of interlaser compensation between these two fluorochromes

no compensation

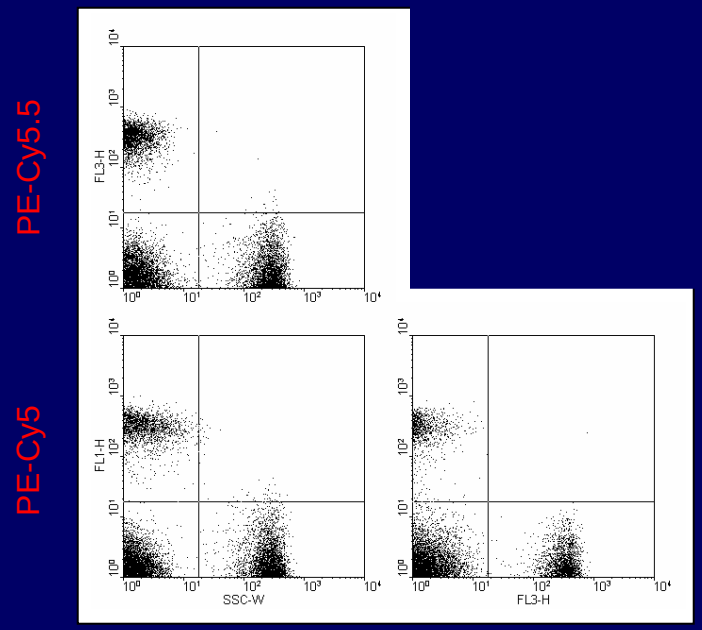
hardware compensation

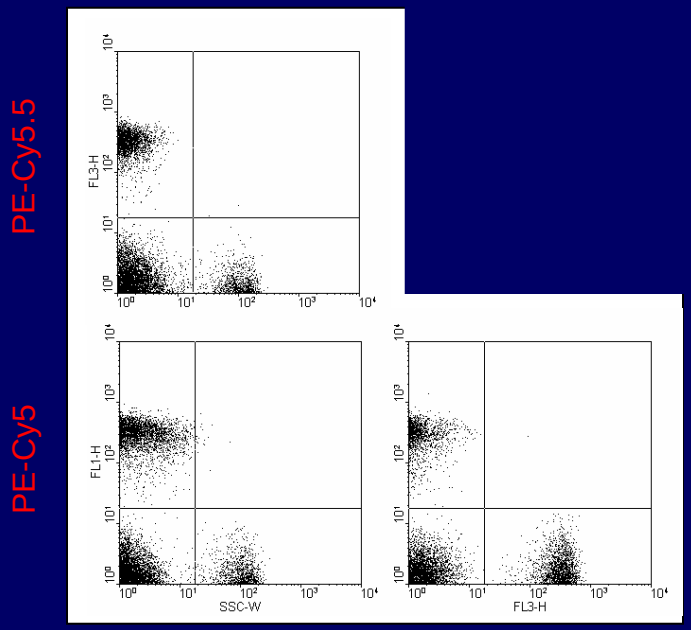


Like PerCP, PerCP-Cy5.5 and PE-Cy5.5 have minimal crossbeam compensation requirements with APC.

PE-Cy7 and PE-Alexa Fluor 750

- PE tandems constructed with the far red Cy dye **Cy7** or the more recent Alexa Fluor 750





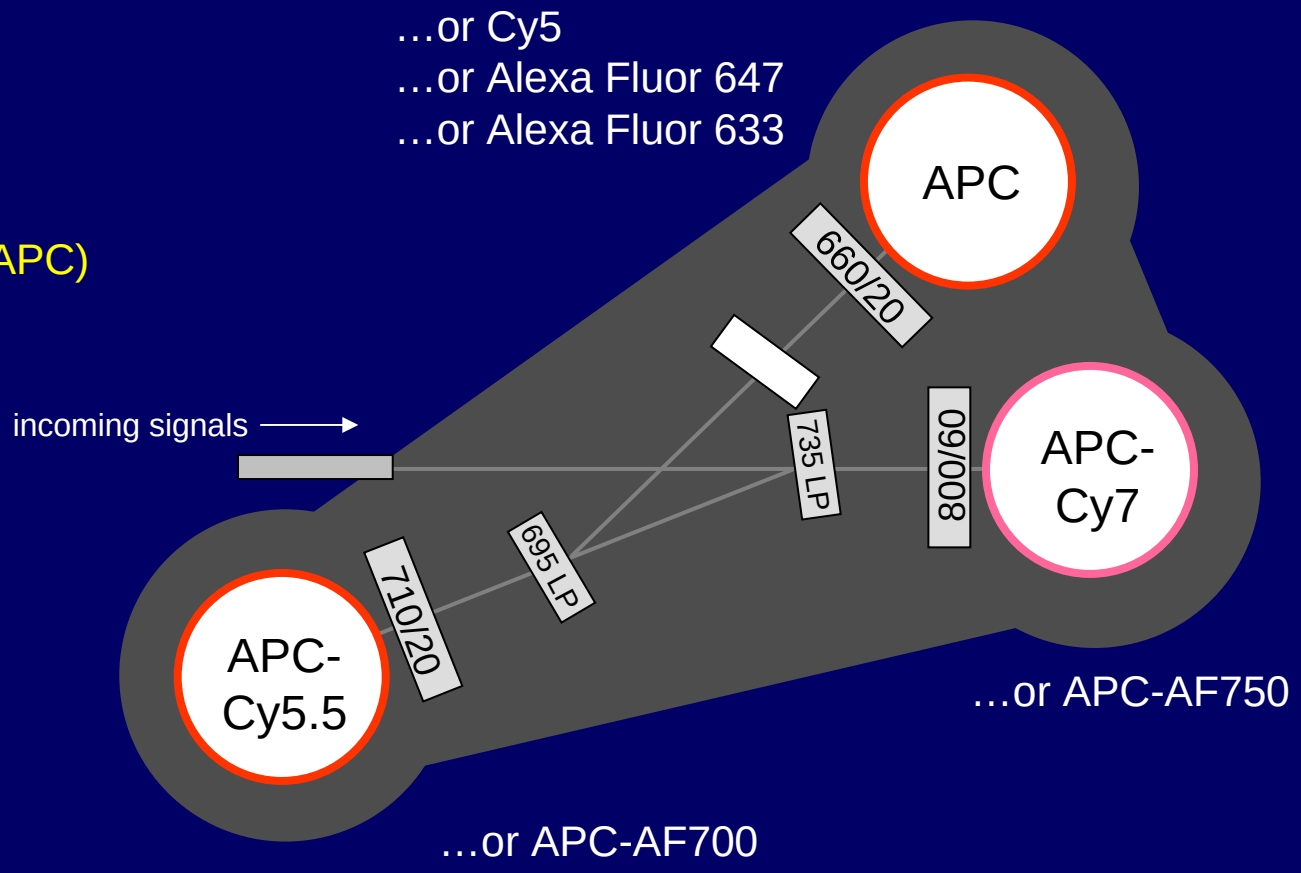
- Energy transfer between PE and Cy7 is less efficient than with PE-Cy5 and PE-Cy5.5 – tandems tend not to be as bright, and more lot-to-lot variation
- Overlap with PE is often significant
- Cy7 is very photosensitive – handling and storage in minimal light

“Typical” detector configuration

BD LSR II

633 nm laser

Allophycocyanin (APC)
APC-Cy5.5
APC-Cy7



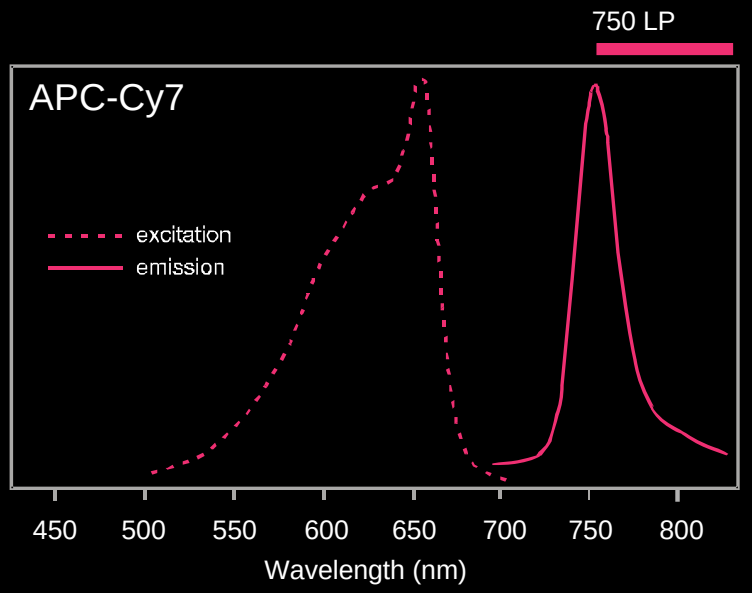
APC tandem conjugates

- APC-Cy5.5
- APC-Alexa Fluor 680
- APC-Alexa Fluor 700

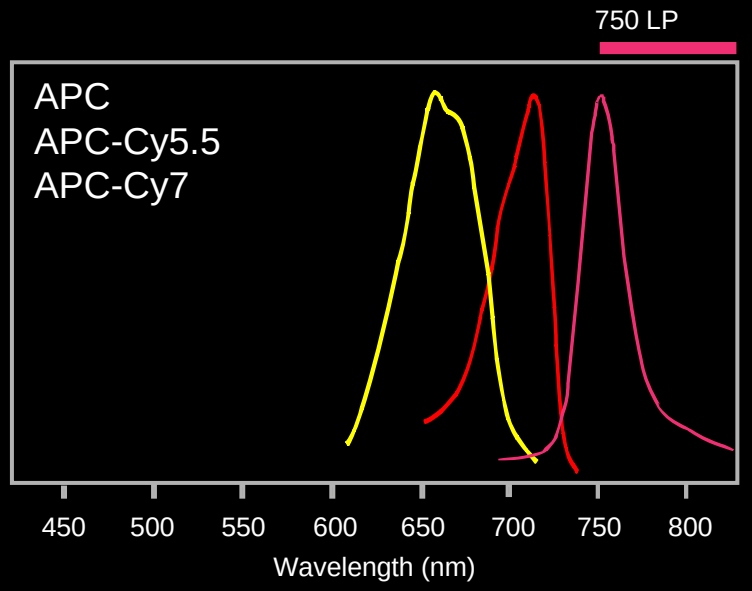
Emit from
680 to 710 nm

- APC-Cy7
- APC-Alexa Fluor 750

Emit from
740 to 800 nm

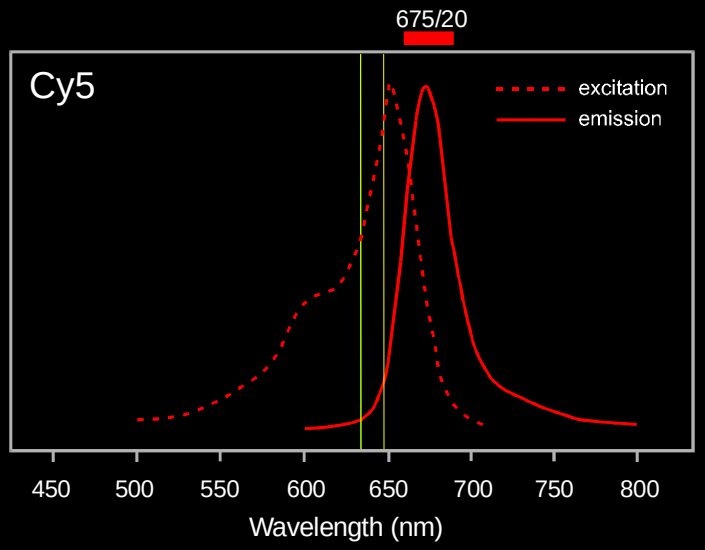


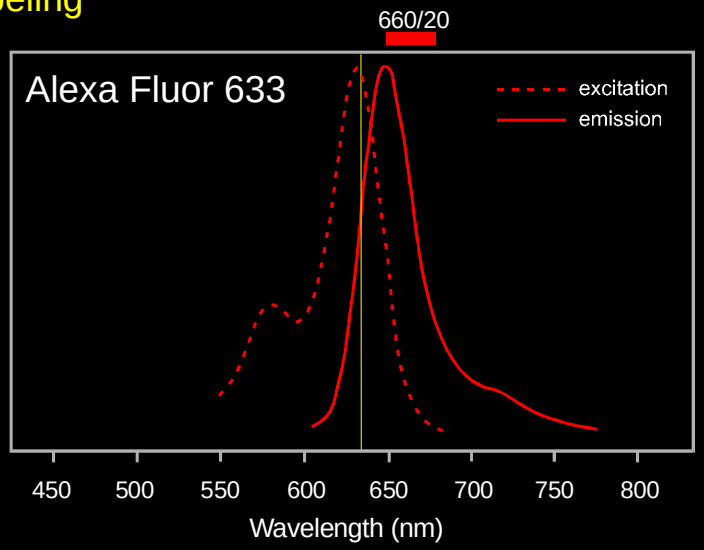
- Analogous emission to their PE counterparts
- Like the PE tandem conjugates, very useful for multicolor flow cytometry in combination with APC and other fluorochromes
- APC and its tandems are also spectrally compatible with each other

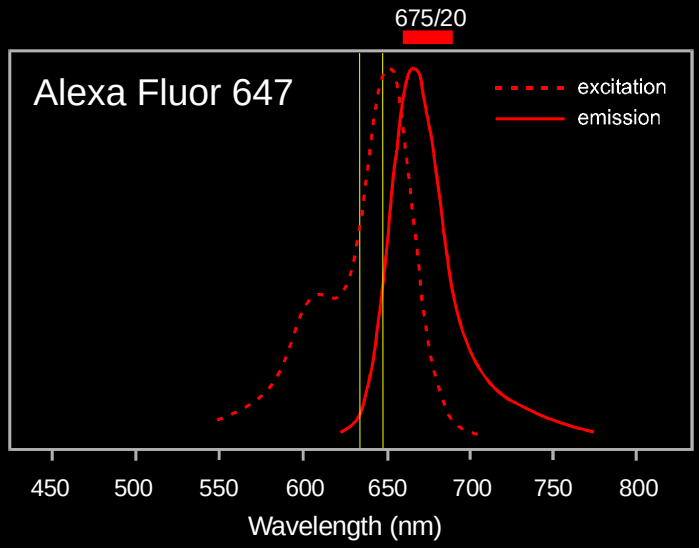


Red-excited low molecular weight fluorochromes

- Red-excited low molecular weight fluorochromes are valuable fluorescent probes for both flow and image cytometry
- Well-excited by red laser sources, with similar (Alexa Fluor 633) or slightly longer (Cy5 and Alexa Fluor 647) emission wavelengths than APC
- All can be readily conjugated to proteins, and Alexa Fluor 647 now available in many direct conjugates
- Non-tandem Alexa Fluor 680 and 700 have also been used with red laser excitation for multicolor labeling

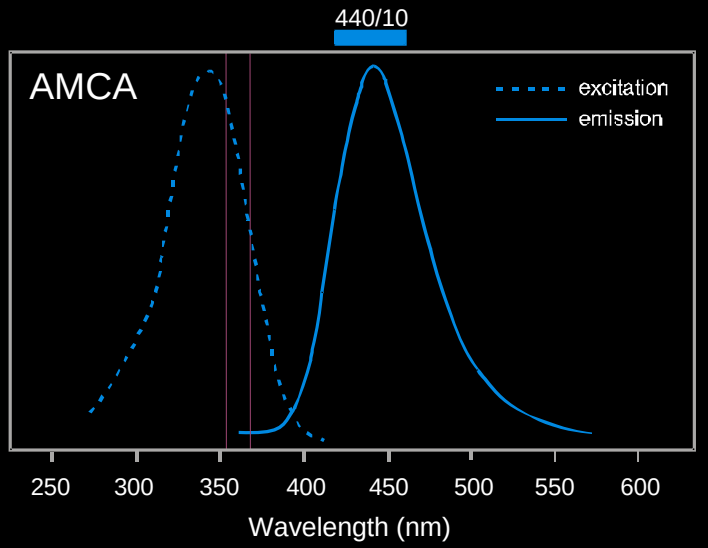


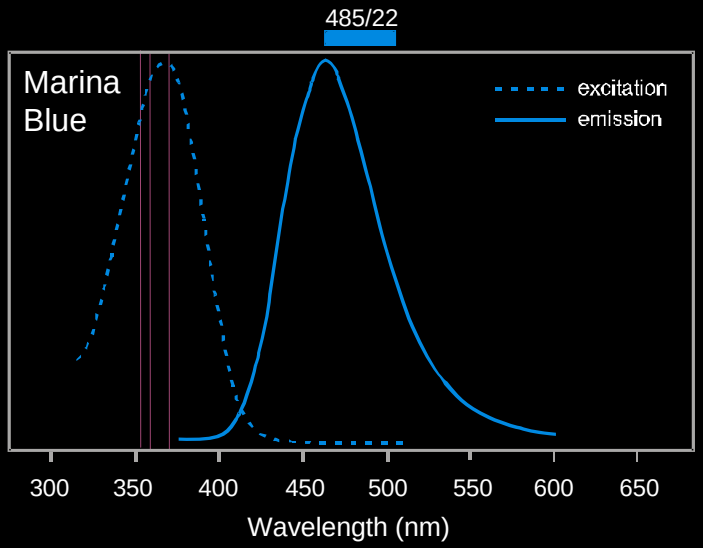


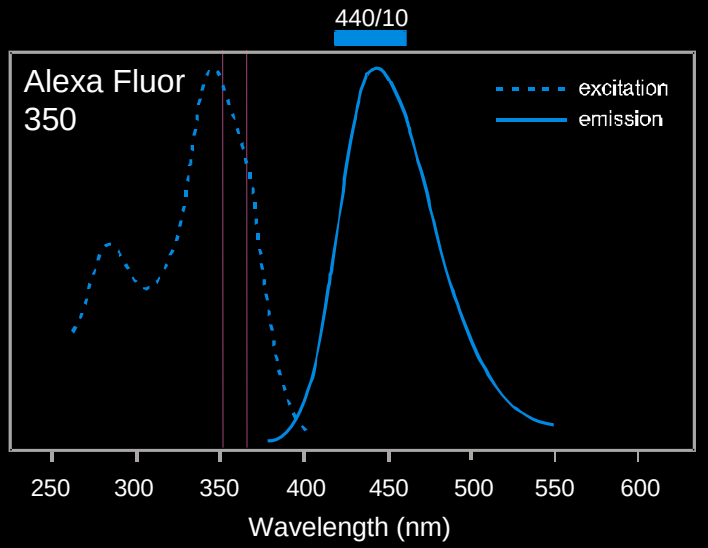


Ultraviolet-excited fluorochromes

- Most are coumarin derivatives
- **Not commonly used in flow cytometry. Why?**
  - Their excitation maxima fall in a region of strong cellular autofluorescence (~450 nm) – signal-to-noise ratio of surface marker labeling tends to be low
  - UV laser sources uncommon and, until recently, difficult to integrate into benchtop instrumentation

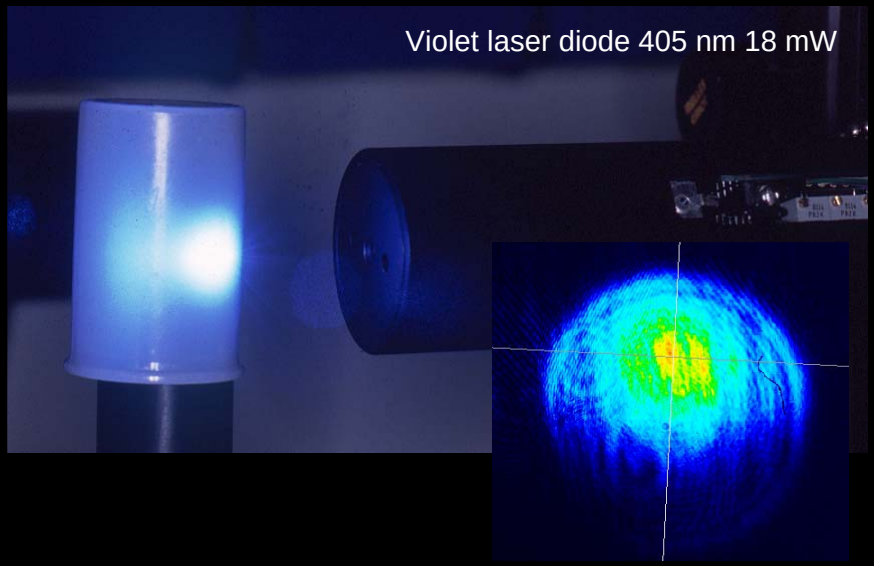


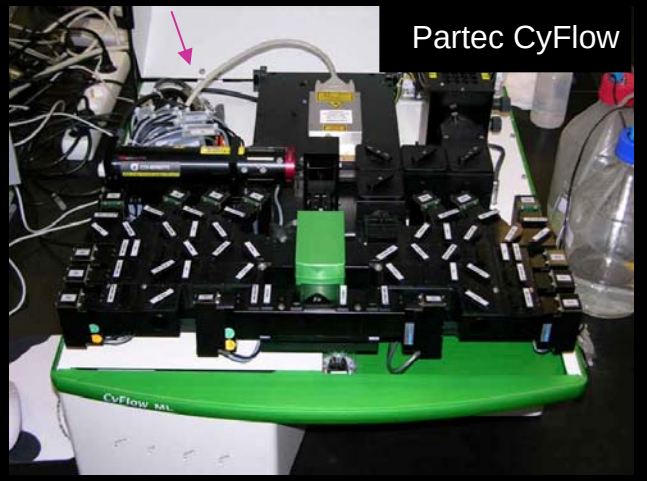




Violet lasers on benchtop flow cytometers

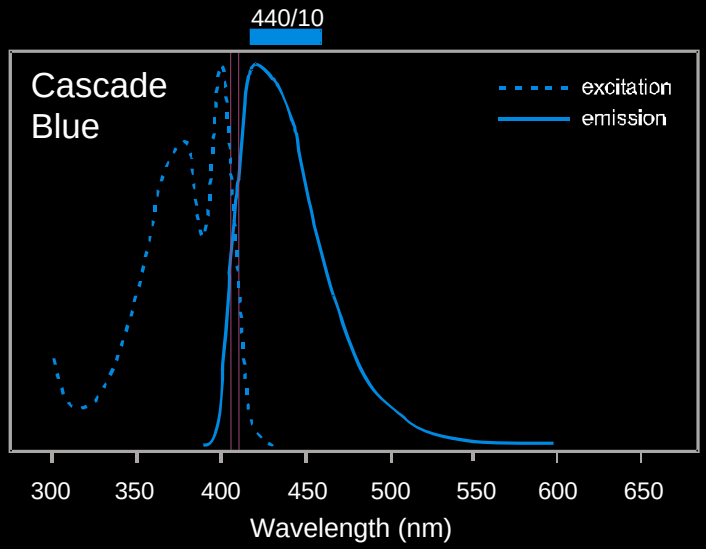
- Solid-state violet laser diodes (VLDs) are now standard equipment on a wide variety of flow cytometers
  - BD LSR II and FACSAria
  - DakoCytomation CyAn
  - Compucyte LSC2 and iCys
  - Partec CyFlow
- These small, reliable, and relatively cheap laser sources have broadened the use of violet-excited fluorochromes such as DAPI, Cascade Blue and Pacific Blue.

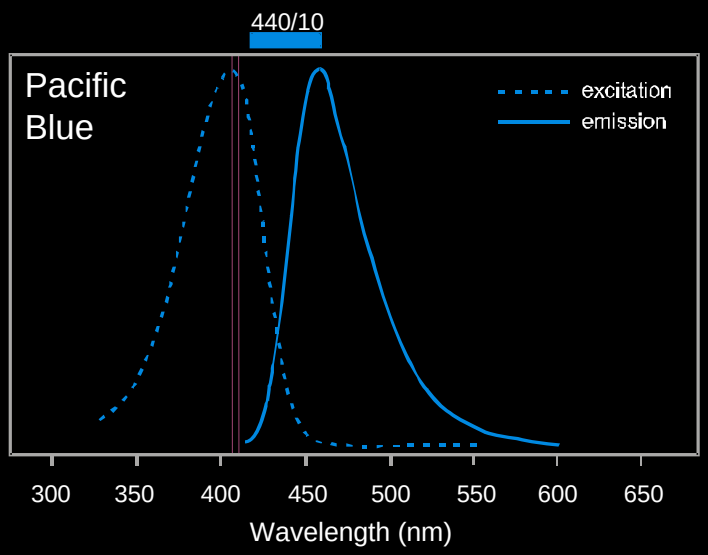


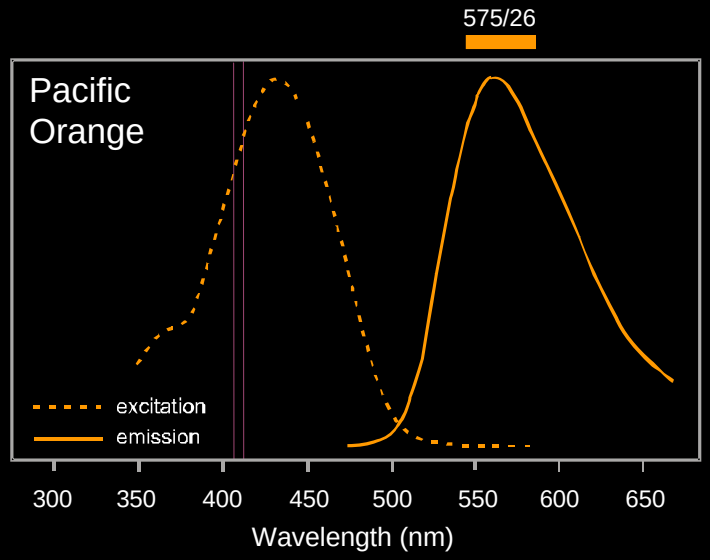


Violet-excited fluorochromes

- Cascade Blue, Alexa Fluor 405 and Pacific Blue are important fluorochromes in polychromatic flow cytometry. Quantum yields similar to fluorescein.
- Cascade Yellow and, Alexa Fluor 430 and AmCyan have been developed as violet-excited, green-emitting fluors for use in combination with Cascade or Pacific Blue. All have technical problems.
- Pacific Orange now a usable second violet fluorochrome

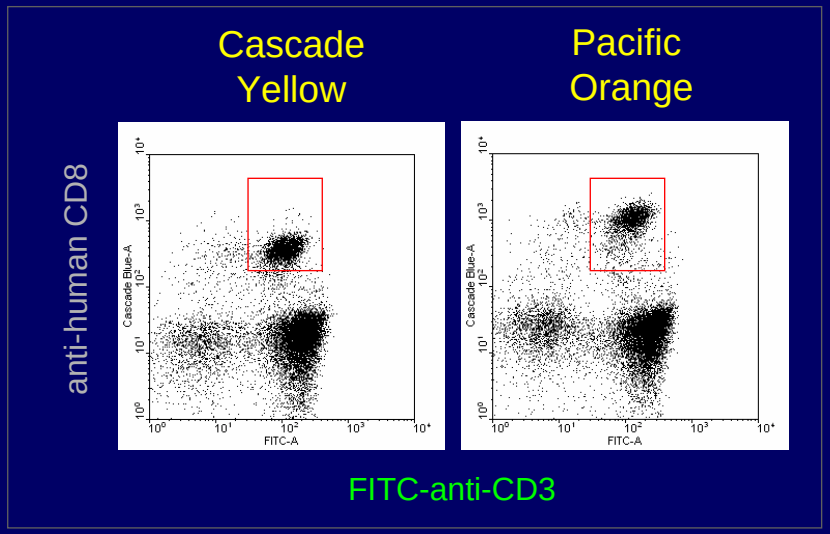


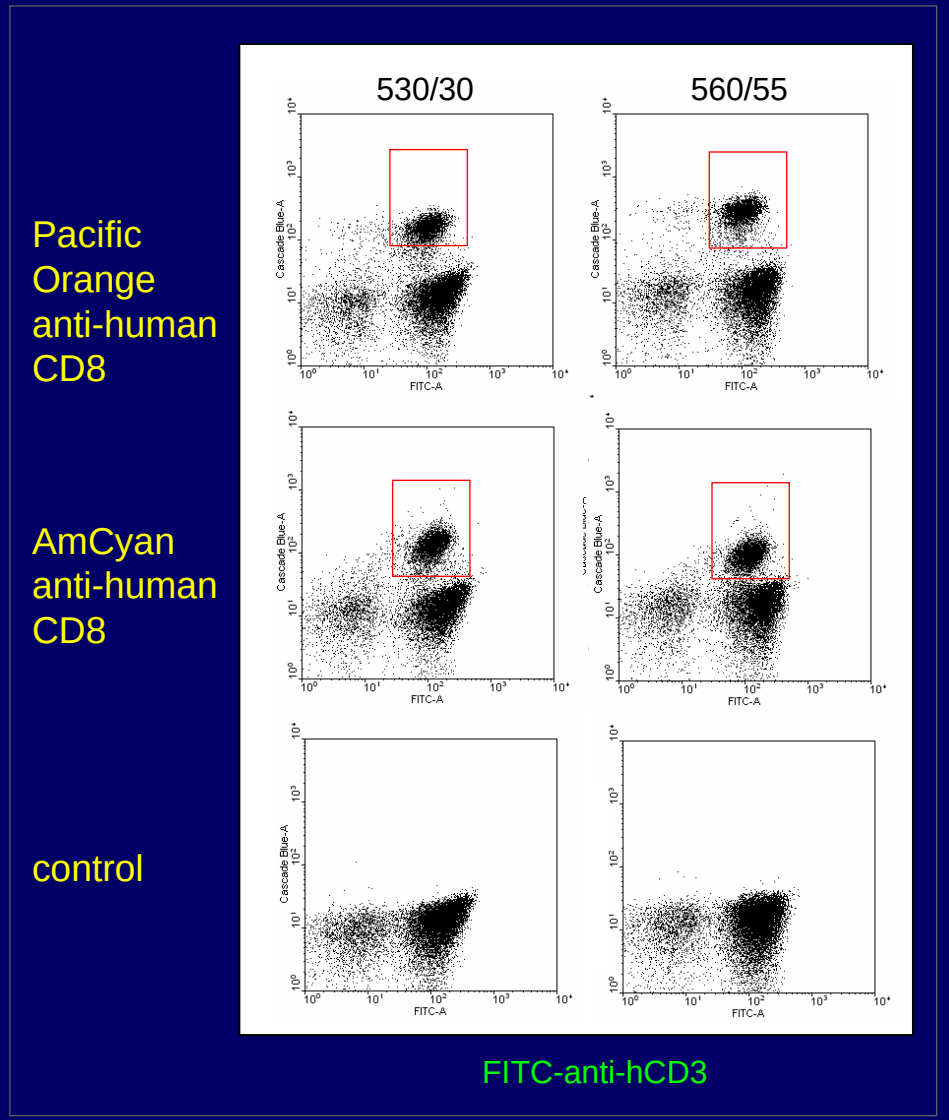




Pacific Orange

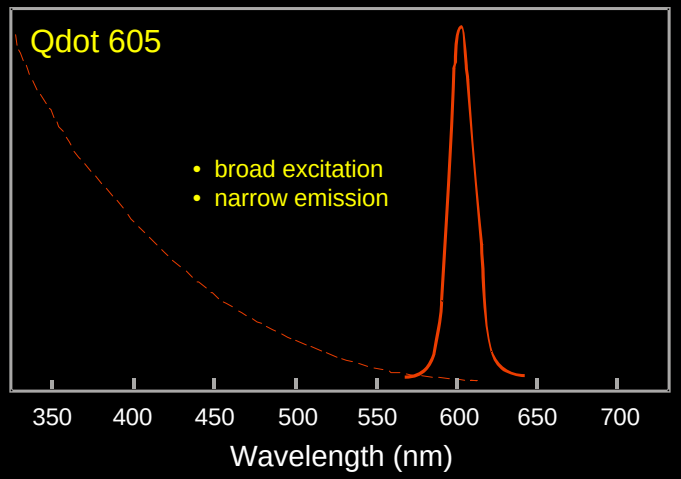
- A brighter replacement for Cascade Yellow and Alexa Fluor 430
- Emits in the PE range (a 575 or 585 nm filter will work fine)
- Spectrally compatible with Alexa Fluor 405, Cascade Blue and Pacific Blue

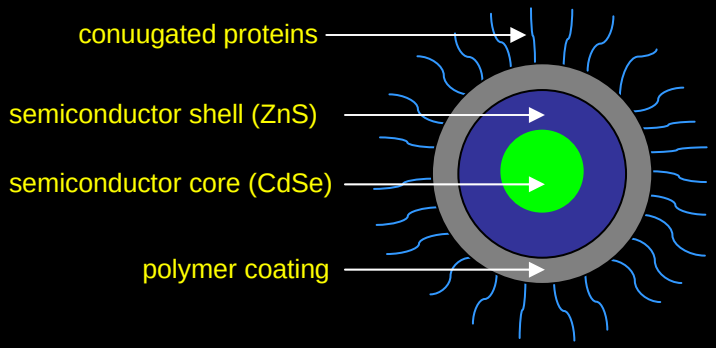


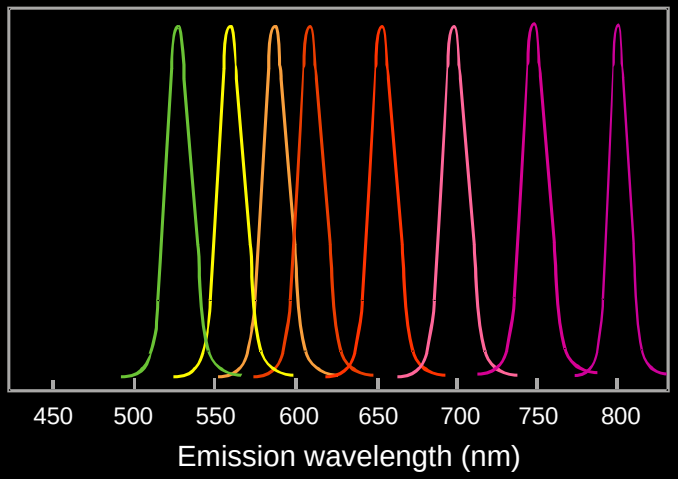


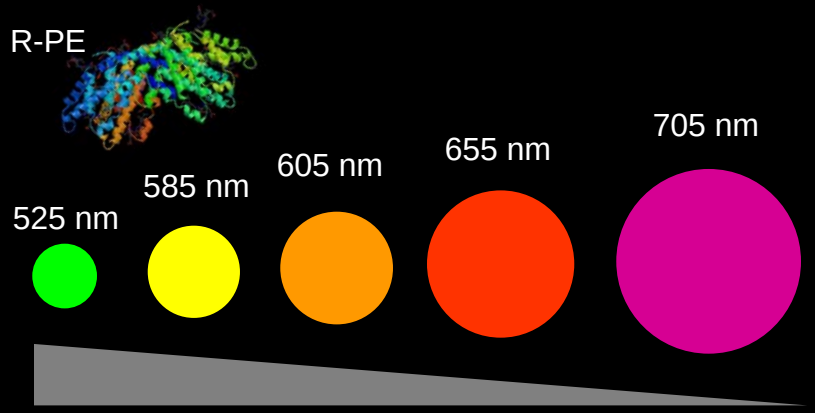
Quantum dots

- Encacsulated semiconductor nanocrystals that fluoresce strongly at tightly defined wavelengths depending on crystal diameter
- Extremely resistant to photobleaching









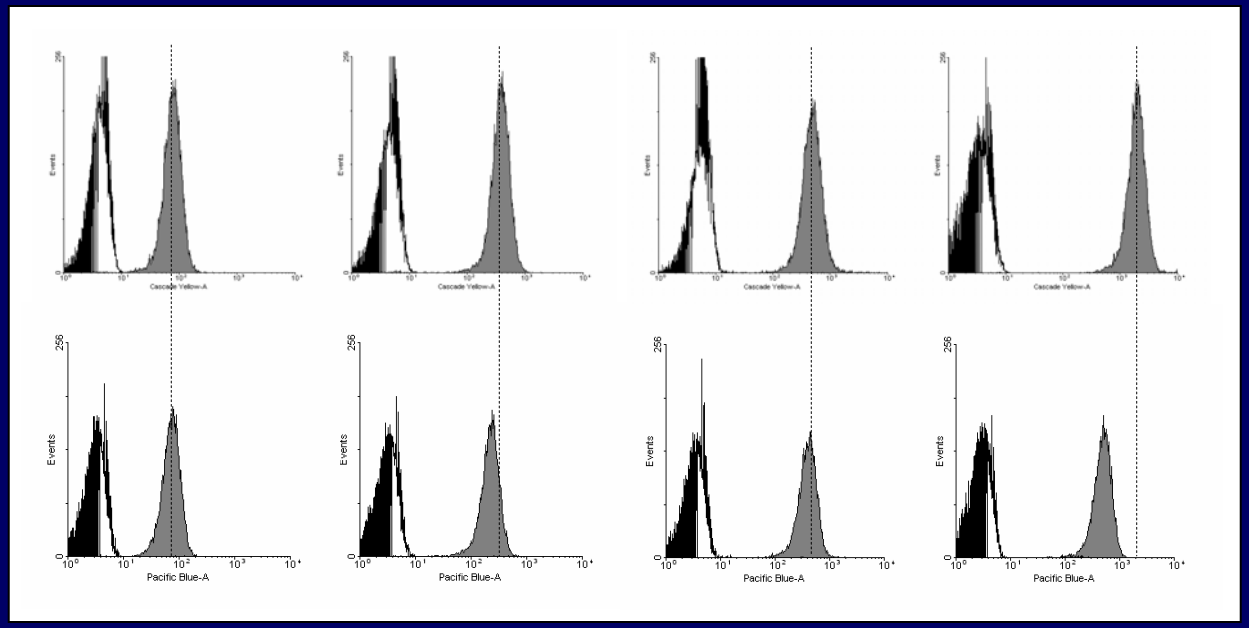
Quantum dots

ZnS:CdSe quantum nanoparticles are best excited with ultraviolet sources (shorter wavelengths better), but can be excited with any excitaton source lower than its emission bandwidth.

Qdot 525 Qdot 585 Qdot 605 Qdot 655

NUVLD
374 nm
10 mW
LSR II

Violet diode
408 nm
22 mW
LSR II



CD44 fluorescence

Violet laser diodes provide an excellent excitation source for quantum nanoparticles.

Quantum dots by flow and image cytometry

LSR II
NUVLD

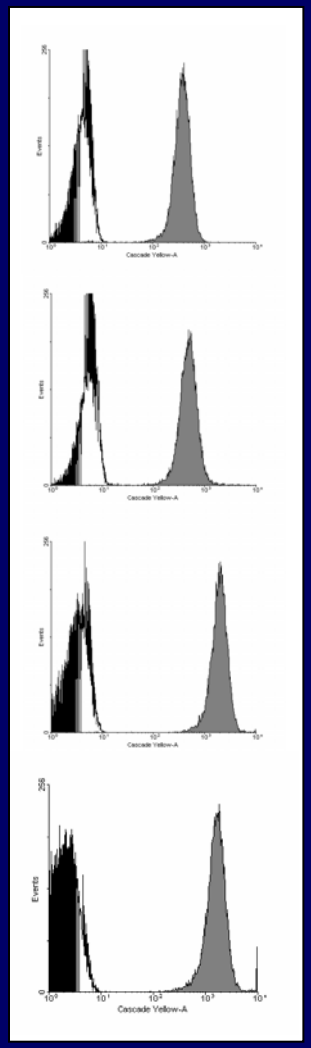
LSC
violet diode

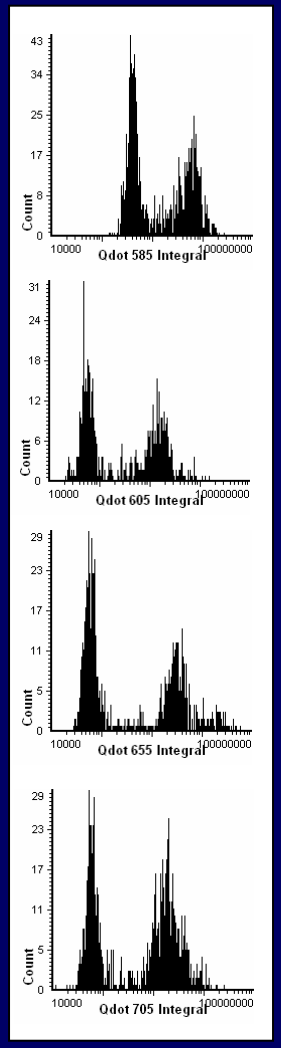
Qdot 585

Qdot 605

Qdot 655

Qdot 705

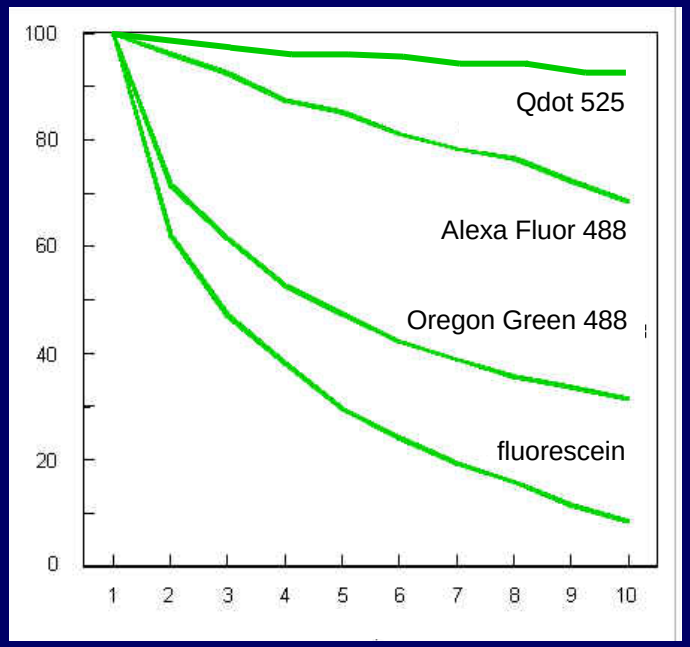




CD44 expression

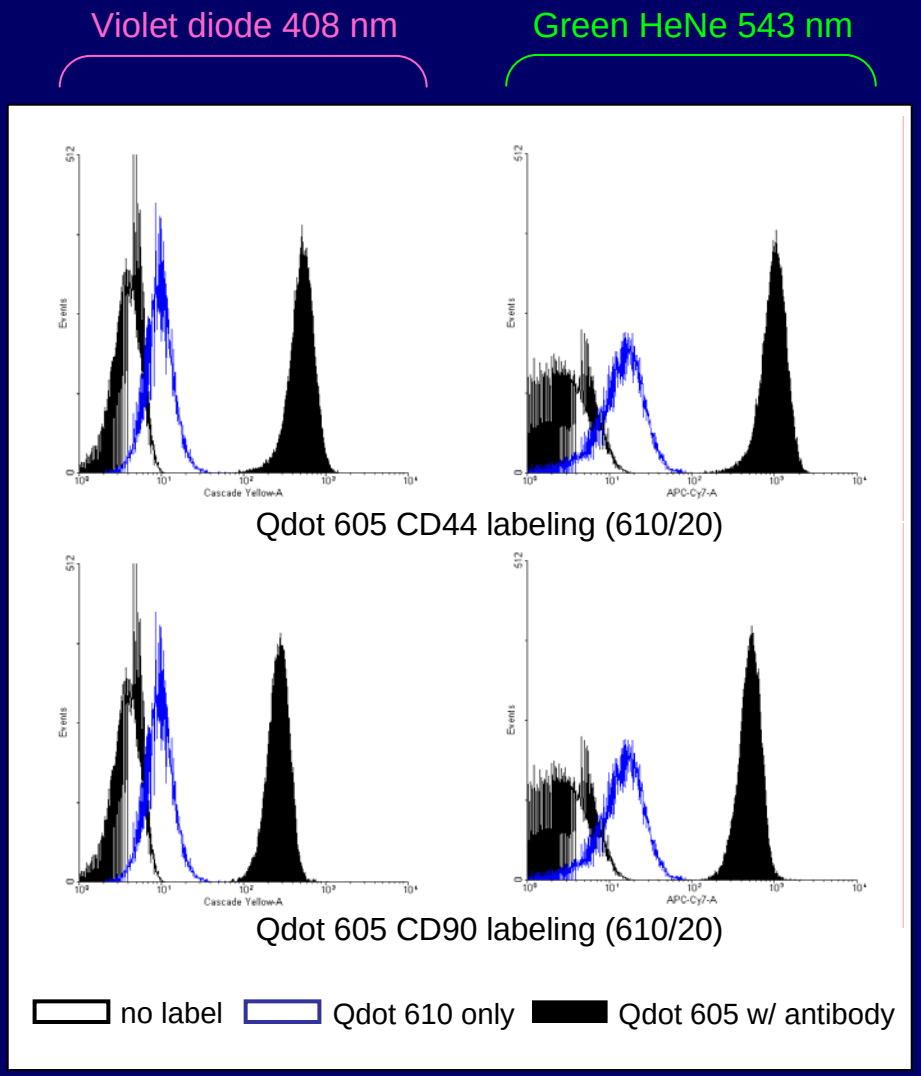
Quantum dots, as inorganic complexes, show virtually no photobleaching upon long-term exposure to light (hence their popularity as confocal fluors)

This photostability makes then especially useful for laser scanning cytometry (although their long fluorescence lifetimes make them useful for flow as well)



Quantum dots

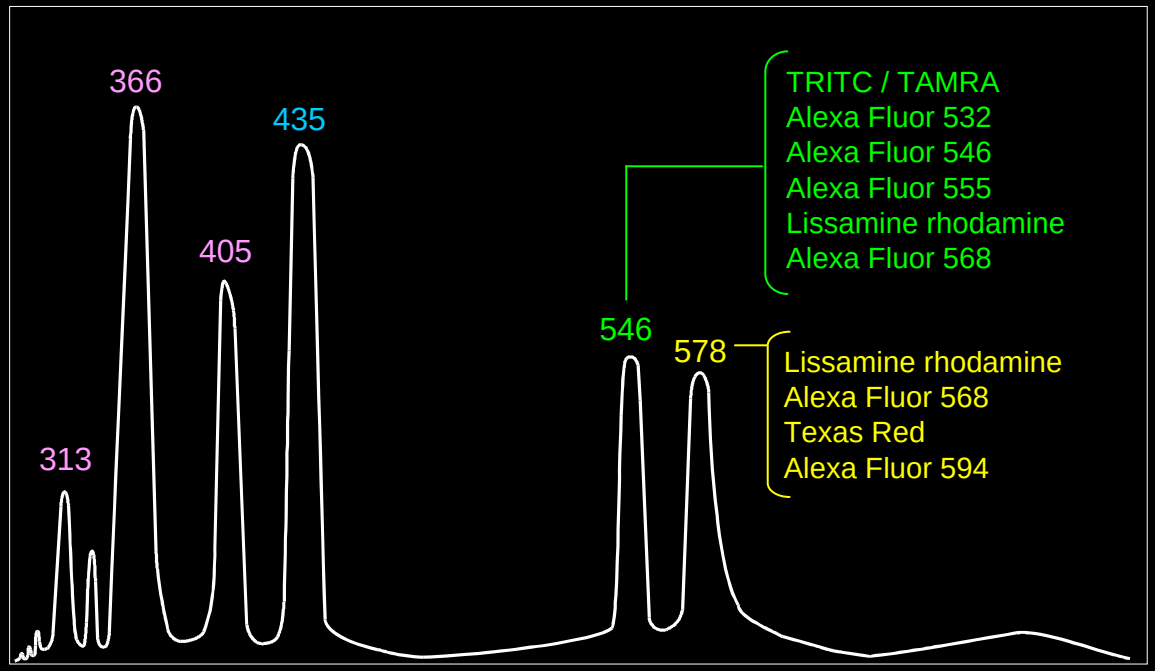
- Because quantum dots have a broad excitation bandwidth they can be excited by virtually any laser wavelength shorter than their emission (shown at left for Qdot 605)
- This can make multilaser flow cytometry with quantum dots problematic



Interchangeability of fluorochromes for confocal imaging and cytometry

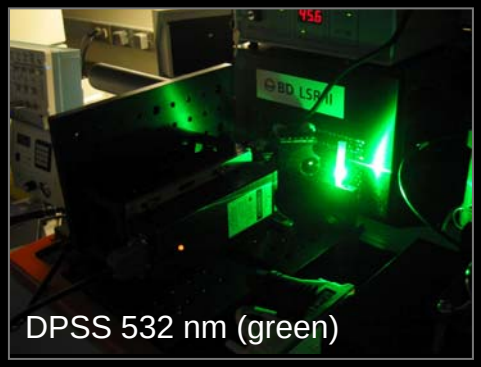
A number of excellent fluors are available with green to yellow excitation bandwidths.

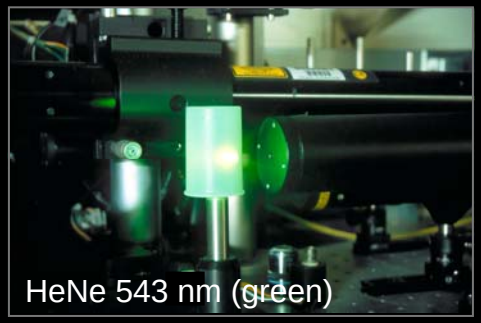
They have high quantum efficiency, and excite/emit in areas of low cellular autofluorescence.

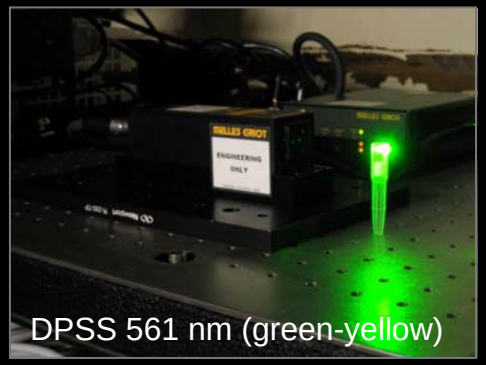


In the past, their rare appearance in cytometry was mainly due to a dearth of laser excitation options at these wavelengths.

Laser sources for green and yellow excited fluorochromes


DPSS 532 nm (green)


HeNe 543 nm (green)


DPSS 561 nm (green-yellow)

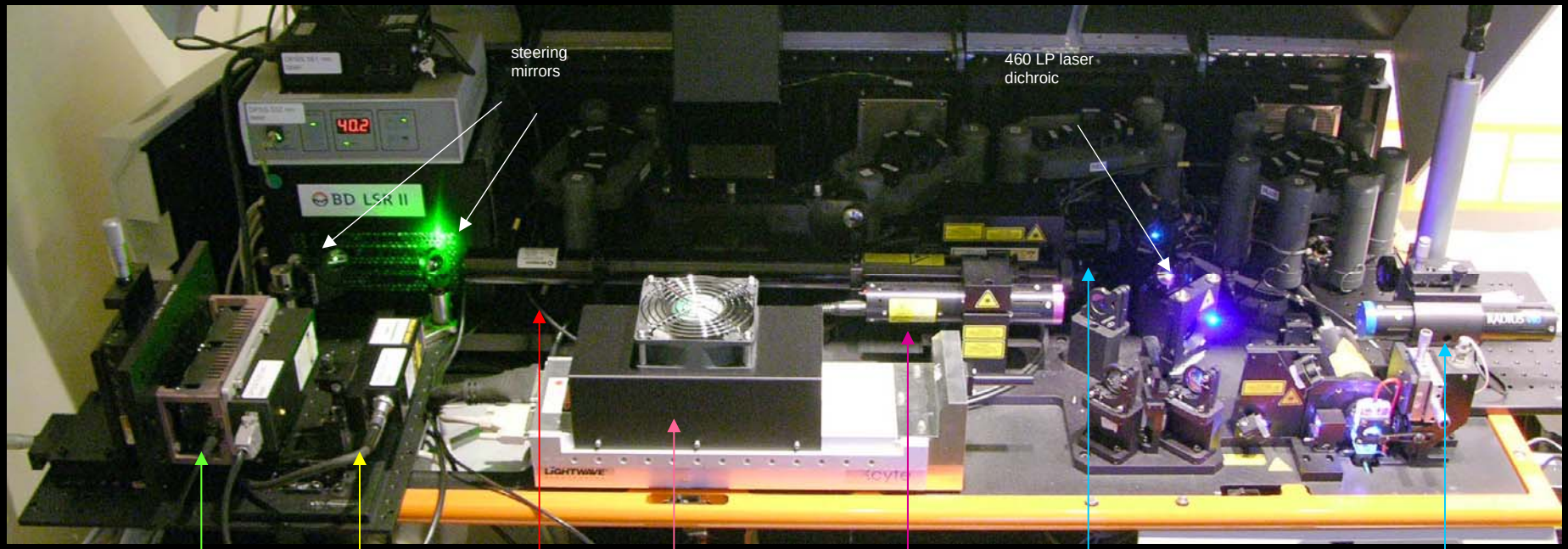

HeNe 594 nm (yellow)

DPSS 532 nm DPSS 561 nm	→	R-phycoerythrin and its tandems B-phycoerythrin TRITC / TAMRA Alexa Fluor 532 Alexa Fluor 546 Alexa Fluor 555 Lissamine rhodamine Alexa Fluor 568 Texas Red and Alexa Fluor 594
HeNe 594 nm	→	Texas Red Alexa Fluor 594 Alexa Fluor 610 APC and APC tandems

These lasers can be readily Integrated Into a variety of instrument platforms.

Current BD LSR II development platform (NCI)

Up to 9 lasers possible

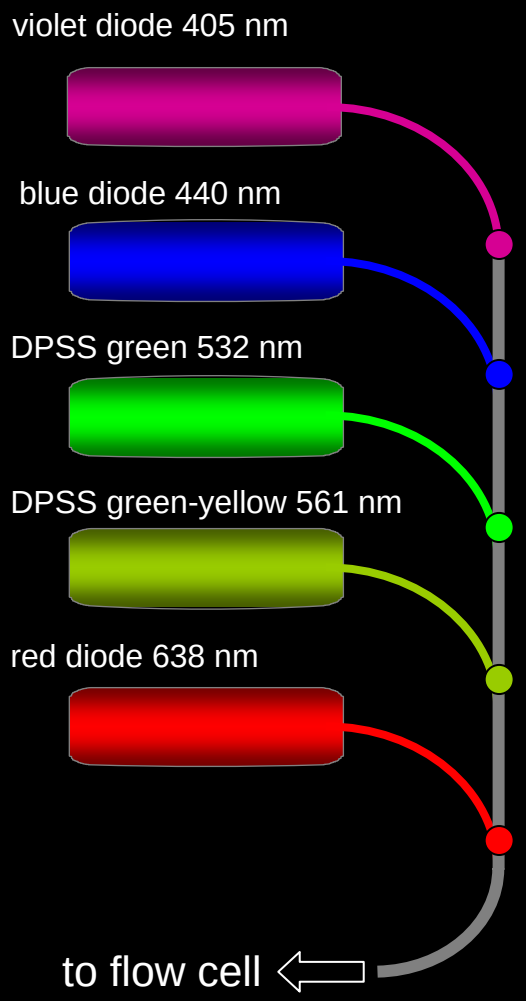


We can excite and use fluorescent probes not normally available for flow cytometry

BD LSR II with fiber optic merge module

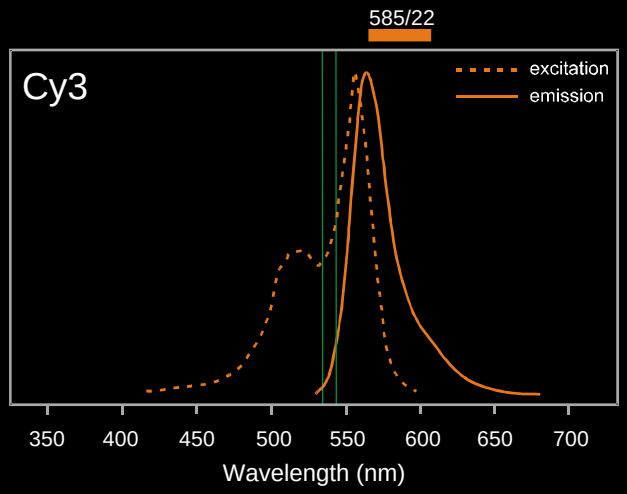
An optical systems where multiple lasers are “merged” into one single mode fiber optic

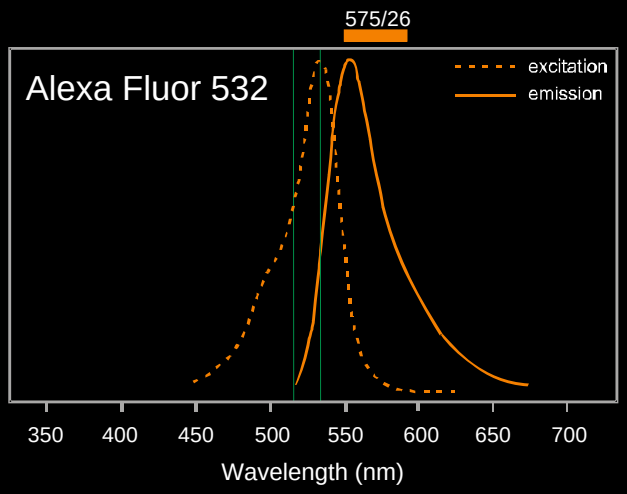
We can switch between laser wavelengths as desired. Useful for incorporating many lasers onto a flow cytometer without significantly modifying its physical configuration.

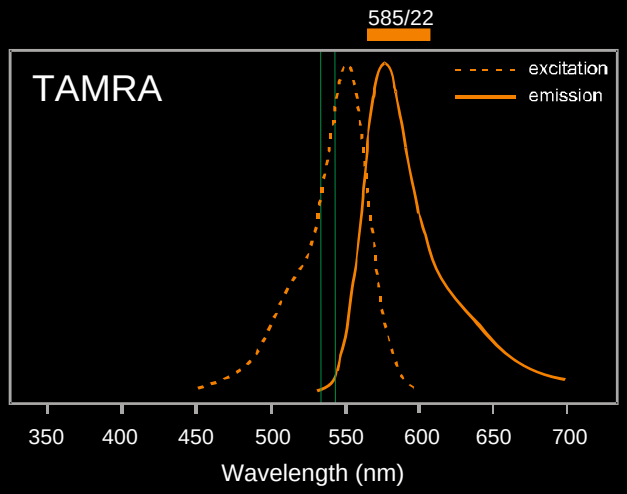


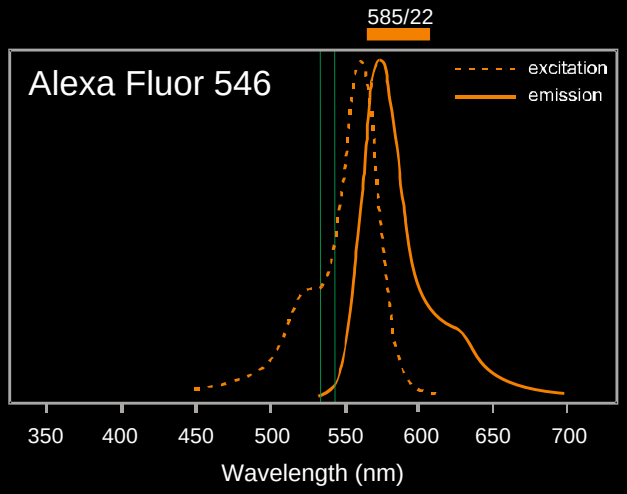
Developed by Spectral Applied Research, Concord, Ontario, Canada

Green-excited low molecular weight fluorochromes



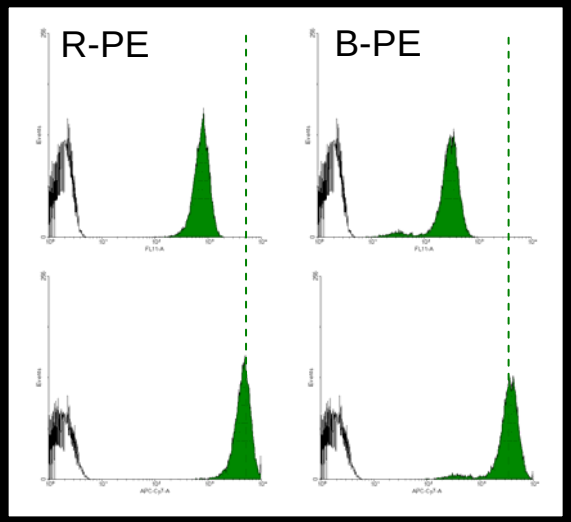






Green lasers

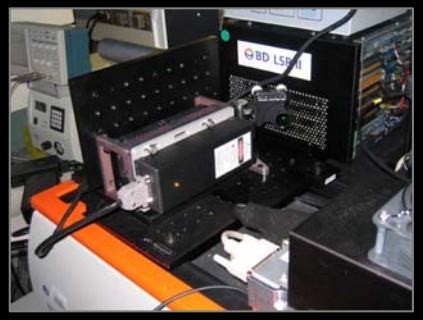
DPSS 532 nm lasers are now widely available, and are commonly integrated into benchtop flow cytometers

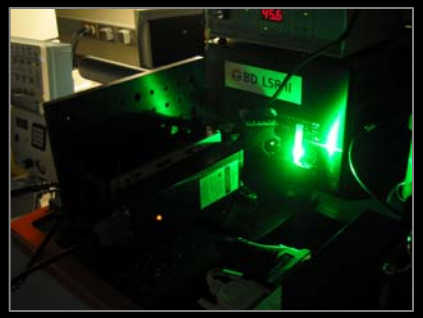


CD44 expression

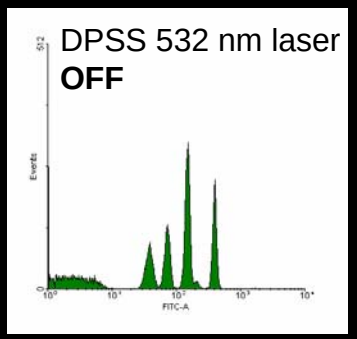
Enhanced excitation and signal-to-noise ratio with **PE and the PE tandems**, and **DsRed**

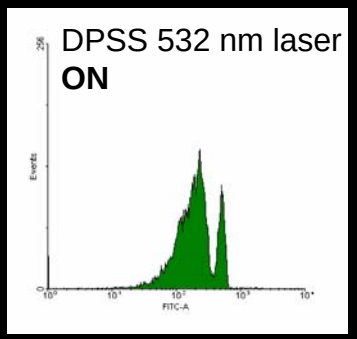
Access to rhodamine-based fluorochromes, and the green-excited Alexa Fluor probes.





Green laser light can “contaminate” the fluorescein detector, requiring some optical modifications

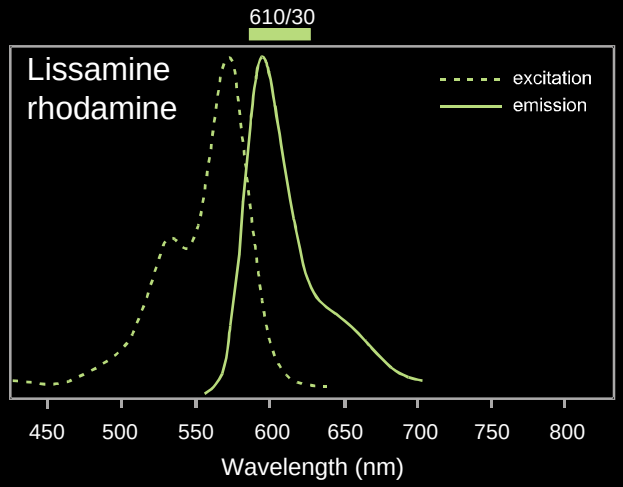


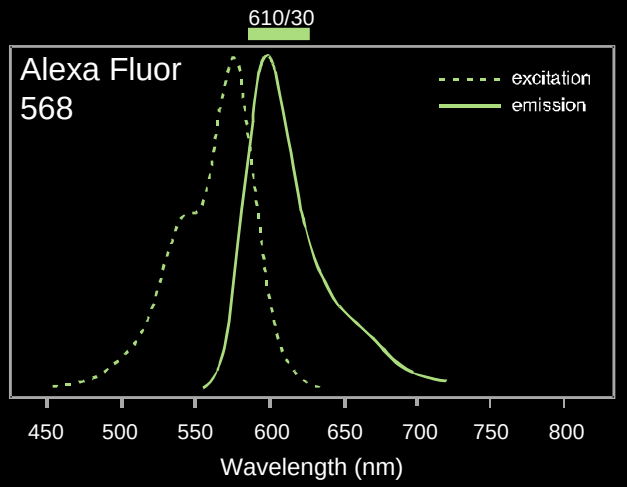


MESF fluorescein sensitivity beads

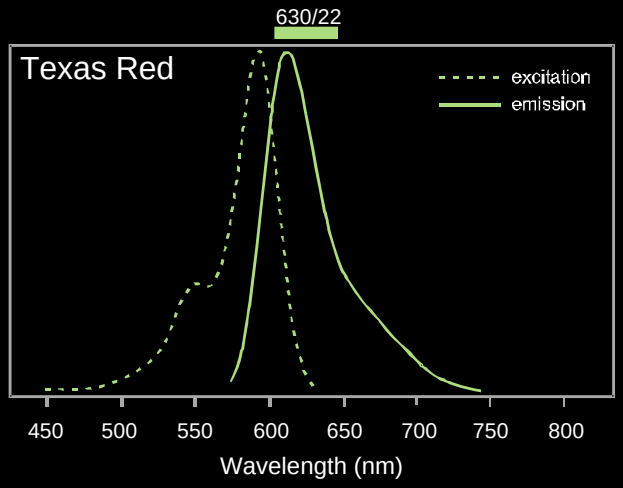
Yellow-excited low molecular weight fluorochromes

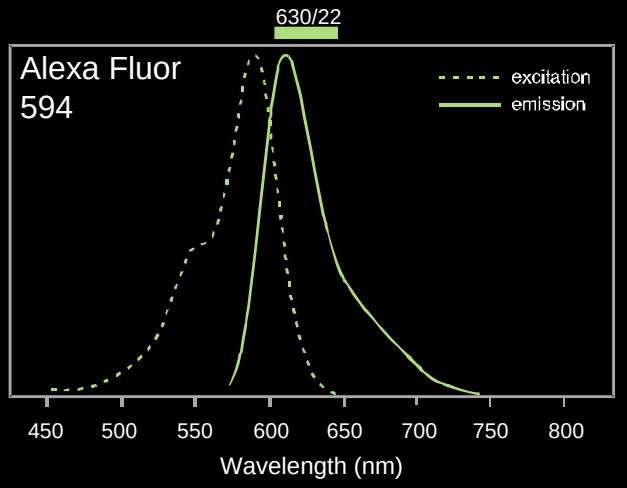
yellow-green excited





yellow-excited



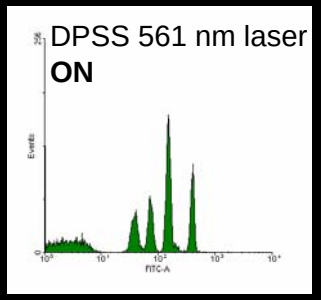


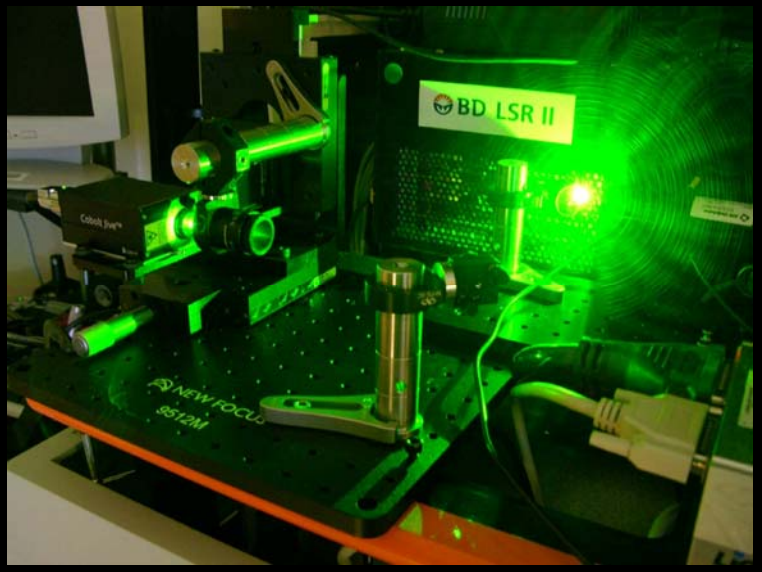
Yellow-excited low molecular weight fluorochromes also make marvelous probes (for the same reason as green) if you have the right laser source.

Green-yellow lasers

DPSS 561 nm lasers are also being integrated into flow cytometers, with power levels up to 50 mW

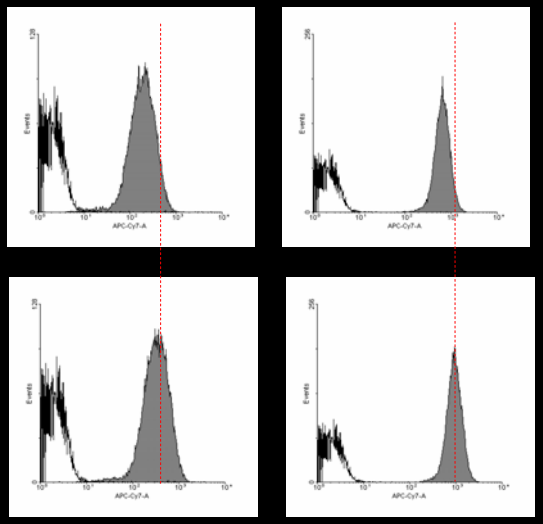
Also excites **PE, PE tandems and DsRed**, with no contamination of the FITC detector





532 nm

561 nm



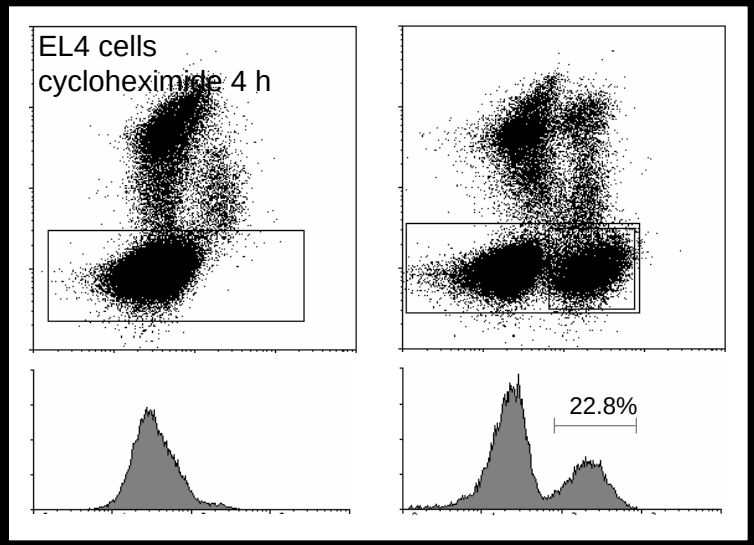
Rhodamine Red CD90

Alexa Fluor 568 CD90

488 nm

561 nm

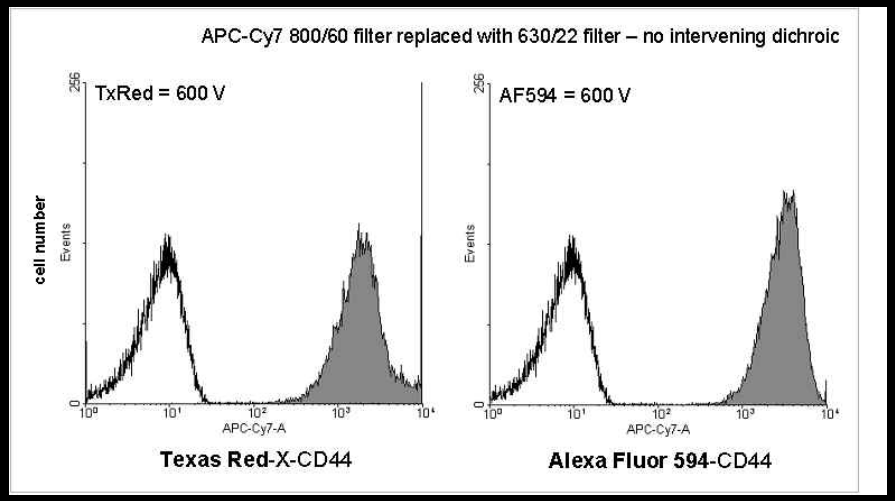
Hoechst 33258



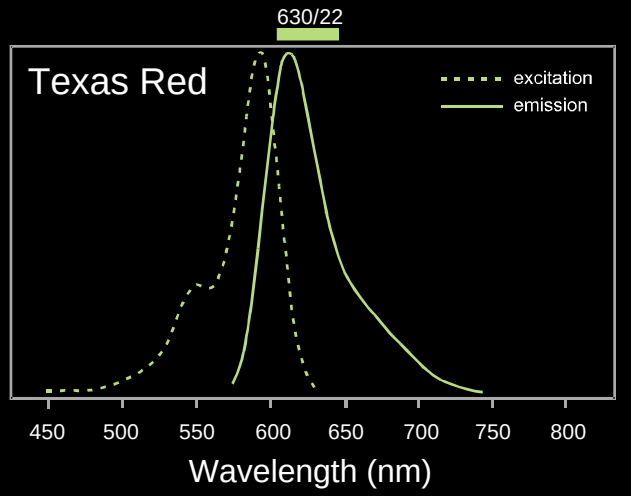
rhodamine caspase 3

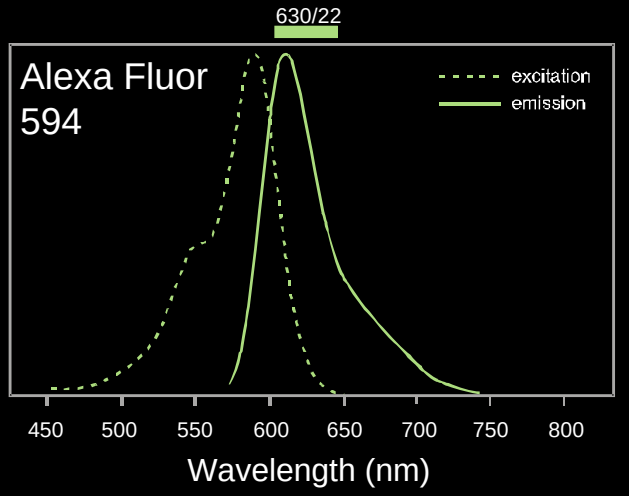
Texas Red and Alexa Fluor 594

- Texas Red was more common in the early days of flow cytometry, but has since suffered from a lack of a practical excitation source
- Yellow HeNe lasers can now be mounted on some flow cytometers, allowing the excitation of Texas Red and Alexa Fluor 594



Using a helium neon 594 nm

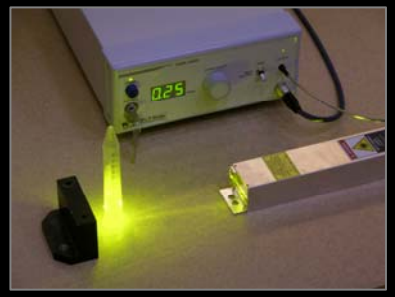




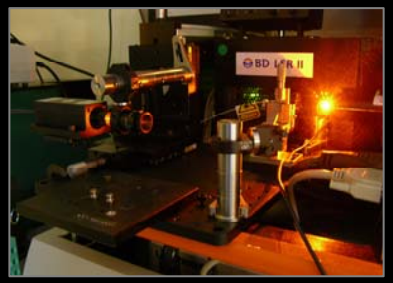
Yellow lasers

Yellow laser light can now be generated in several ways that are compatible with benchtop instrumentation…

Helium neon 594 nm
(up to 7 mW)



DPSS 580 nm fibre-coupled
(up to 800 mW!)



DPSS 593.5 nm
(up to 50 mW)

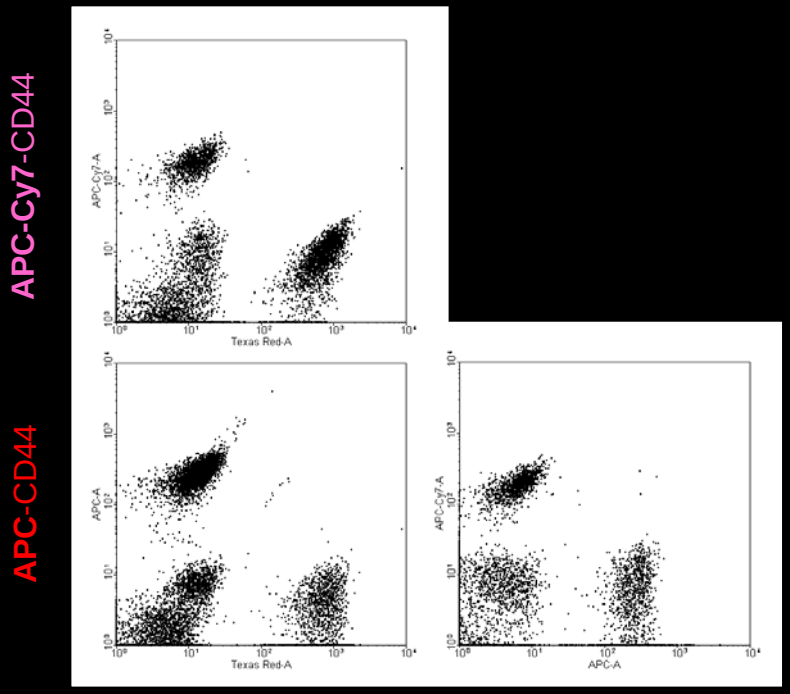
Excellent excitation of Texas Red, Alexa Fluor 594 and Alexa Fluor 610.

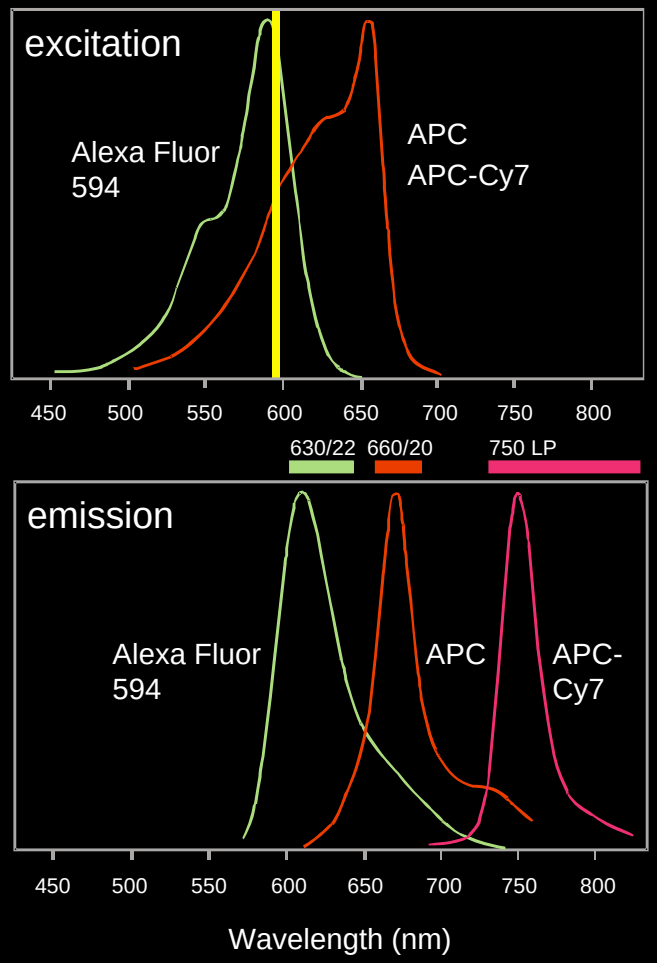
Also good excitation of APC and its tandems. You can replace your red laser with a yellow source for multicolor analysis.

Telford, Poster P-235

Simultaneous excitation of Texas Red or Alexa Fluor 594, APC and APC-Cy7

- Yellow HeNe lasers can excite, Texas Red (or Alexa Fluor 594), APC and the APC tandems
- Up to **four** fluorochromes can be analyzed simultaneously using a yellow HeNe





NCI ETIB Flow Cytometry Core Laboratory

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All handouts and supplementary materials from this Tutorial can be downloaded at:

http://home.ncifcrf.gov/ccr/flowcore/ISAC2006/ISAC2006.htm